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# Disruption of ectoplasmic specializations between Sertoli cells and maturing spermatids by anti-nectin-2 and anti-nectin-3 antibodies

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## Abstract

**Aim:** To understand the biological functions of the ectoplasmic specializations between Sertoli cells and maturing spermatids in seminiferous epithelia. **Methods:** In order to disrupt the function of the ectoplasmic specializations, nectin-2, which is expressed at the specialization, was neutralized with anti-nectin-2 antibody micro-injected into the lumen of the mouse seminiferous tubule. Anti-nectin-3 antibody was also micro-injected into the lumen in order to neutralize nectin-3, which is expressed at the specialization. **Results:** The actin filaments at the specialization disappeared, and exfoliation of maturing spermatids was observed by electron microscopy. **Conclusion:** Nectin-2 was neutralized by anti-nectin-2 antibody and nectin-3 was neutralized by anti-nectin-3 antibody, respectively. Inactivated nectin-2 and nectin-3 disrupted the nectin-afadin-actin system, and finally the actin filaments disappeared. As a result, the specialization lost the holding function and detachment of spermatids was observed. One of the functions of the specialization seems to be to hold maturing spermatids until spermiation. (*Asian J Androl* 2008 Jul; 10: 577–584)

**Keywords:** ectoplasmic specialization; Sertoli cell; spermatogenic cell; testis; actin; nectin; mice

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## 1 Introduction

Two types of ectoplasmic specializations are observed in the seminiferous epithelium. One exists between adjoining Sertoli cells, and the other exists between Sertoli cells and maturing spermatids. Both types of specializa-

tions in the seminiferous epithelium were formerly called junctional specializations. The widely used term “ectoplasmic specializations” was proposed by Russell [1]. The ectoplasmic specialization between adjoining Sertoli cells is equipped with more than 100 tight junctional strands and forms one of the tightest junctions. The junctional strands function as the blood-testis barrier, establishing a specialized environment essential for germ cell development. The strands also function as an immunological barrier, but the function is incomplete [2]. The function of the specialization between the Sertoli cell and the maturing spermatids is not known. Many hypotheses have been proposed: shaping of the sperm

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head; holding maturing spermatids until spermiation; releasing spermatozoa at the time of spermiation; alignment of the maturing spermatids in the seminiferous epithelium; and providing a scaffold for the microtubules.

As unique structures, both types of specializations accompany layers of actin filaments [3] and the subsurface cisternae of the smooth endoplasmic reticulum. Knowledge of the molecules expressing in both specializations is accumulating [4, 5].

Nectins, Ca<sup>2+</sup>-independent immunoglobulin (Ig)-like cell-cell adhesion molecules, play roles in cell adhesion, migration, and polarization [6]. They constitute a family of four members, nectin-1, nectin-2, nectin-3 and nectin-4, and are associated with actin filaments through afadin. Nectin-1, nectin-2 and nectin-3 are ubiquitously expressed in a variety of cells, including fibroblasts, epithelial cells, and neurons [7]. Nectin-2 and nectin-3 are also expressed in cells that lack cadherins, such as B cells and monocytes. Human nectin-4 is expressed mainly in the placenta. Nectin-1 and nectin-2 were originally isolated as poliovirus receptor-related proteins and named PRR-1 and PRR-2, respectively. They were later renamed HveC and HveB, respectively.

Nectins have an extracellular domain containing three Ig-like loops, a transmembrane domain and a cytoplasmic tail domain. Furthermore, the tail domain has a conserved motif of four amino acid residues (Glu/Ala-X-Tyr-Val) at the carboxyl terminus and this motif binds the PDZ domain of afadin. Actin filaments bind the F-actin-binding domain in afadin [7], forming the nectin-afadin-F-actin system.

Sertoli cells express nectin-2 [8–10] and spermatids express nectin-3 [9, 11]. In Sertoli cells, two nectin-2 molecules form a homo-*cis*-dimer, whereas, in the spermatids, two nectin-3 molecules form a homo-*cis*-dimer [6]. At the Sertoli cell-maturing spermatid ectoplasmic specialization, the nectin-2 dimer in the Sertoli cell and the nectin-3 dimer in the spermatid form a hetero-*trans*-dimer that is much stronger in binding than the homo-*trans*-dimer [6].

In the present study, nectin-2 in the Sertoli cells was inactivated by anti-nectin-2 antibody. Similarly, nectin-3 in the spermatids was inactivated by anti-nectin-3 antibody. In both experiments, the Sertoli-maturing spermatid ectoplasmic specialization was disrupted and maturing spermatids were exfoliated from the seminiferous epithelium. The Sertoli-Sertoli ectoplasmic specializations, however, were not affected.

## 2 Materials and methods

Adult ICR mice were used in this study. Animal handling was approved by the Animal Research Committee of Chiba University (Chiba, Japan).

### 2.1 Immunohistochemistry

Animals were anesthetized with pentobarbital and fixed with Bouin's fluid by perfusion through the left ventricle. The testes were removed, immersed in the same fixative, processed for paraffin embedding and cut at a thickness of 5  $\mu$ m. Sections were autoclaved at 120°C for 5 min to activate the antigen. Non-specific binding of the antibody was blocked in 10% fetal bovine serum in phosphate-buffered saline (PBS) for 30 min at room temperature. The sections were then incubated with goat anti-nectin-2 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The antibody was raised against a peptide mapping near the N-terminus in the extracellular domain. After washing with PBS, samples were incubated with biotinylated rabbit antigoat IgG (1:500 dilution; Dako Japan, Kyoto, Japan) for 1 h, followed by incubation with streptavidin-biotin peroxidase complex solution (DAKO LSAB2 system; Dako, Japan) for 30 min. Immunohistochemical reactions were visualized using 3, 3'-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub>. The sections were then counterstained with hematoxylin. Sections for the negative control were processed in the same manner, but the anti-nectin-2 antibody treatment was omitted.

### 2.2 Confocal laser scanning microscopy

Animals were perfused through the left ventricle with 4% paraformaldehyde in PBS. The testes were removed, immersed in the same fixative for an additional 4 h, rinsed with PBS, embedded in optimal cutting temperature compound and cut at a thickness of 20  $\mu$ m on a cryostat. The sections were incubated with a blocking solution for 1 h at room temperature to block the non-specific binding of the antibody, then incubated with rat anti-nectin-3 antibody (1:500 dilution; Abcam, Cambridge, UK) at 4°C overnight. The antibody was raised against the recombinant fragment corresponding to the mouse nectin-3 extracellular domain. After washing with PBS, the slides were incubated with Alexa Fluor 488 goat antirat IgG (0.5  $\mu$ g/mL; Invitrogen, Carlsbad, CA, USA) and propidium iodide (1  $\mu$ g/mL; Sigma, St. Louis, CA, USA) in the blocking solution for 1 h at room temperature. After washing with PBS, the samples were mounted with PermaFluor

(Immunon, Pittsburgh, PA, USA) and observed using a confocal laser scanning microscope (LSM 410; Zeiss, Jena, Germany). Sections processed in the absence of the anti-nectin-3 antibody were used for the negative control.

### 2.3 Micro-injection experiments

Anti-nectin-2 antibody, anti-nectin-3 antibody and anti-afadin antibody were used in the micro-injection experiments. Ten microliters of the antibody solution, 890  $\mu$ L of medium CZB [12], and 100  $\mu$ L of 1% Trypan blue was mixed thoroughly and filtered with a Millipore filter (0.22  $\mu$ m; Millipore, Bedford, MA, USA). The solution contained 0.01% sodium azide.

The mixture was micro-injected into the lumen of the seminiferous tubule according to the method introduced by Brinster and Zimmermann [13] and Koh *et al.* [14]. Briefly, the scrotum and tunica vaginalis were cut with surgical scissors under deep anesthesia. The tunica albuginea was exposed and a small cut (approximately 0.1 mm in length) was made in the tunica. A glass pipette (inner diameter, 70  $\mu$ m) filled with the antibody mixture was inserted into the lumen of the seminiferous tubule through the cut in the tunica. Approximately 3  $\mu$ L of the mixture was injected into the lumen. The topography of the seminiferous tubules showing blue in color, because of the Trypan blue in the mixture, was recorded, and the tunica vaginalis and the scrotum were sutured. One seminiferous tubule in the right testis was selected for the micro-injection. The left testis remained intact. Antibodies against nectin-2, nectin-3, and afadin were micro-injected.

After 1, 2, 5 and 12 h, the animals were anesthetized and perfusion fixed with glutaraldehyde. The testes were processed for electron microscopy. Semithin sections stained with toluidine blue were observed with a light microscope, and ultrathin sections stained with uranyl acetate and lead citrate were observed with an electron microscope (JEM 1200EX II; JEOL, Tokyo, Japan). As the seminiferous tubule often makes hairpin bends in the course, it is common to observe several cross-sections of a seminiferous tubule in a single histological section.

As negative control experiments, the same amounts of the mixtures without the antibody were micro-injected into the seminiferous tubules. Briefly, 10  $\mu$ L of the antibody solution without antibody, 890  $\mu$ L of medium CZB, and 100  $\mu$ L of 1% Trypan blue was mixed thoroughly and filtered with a Millipore filter (0.22  $\mu$ m). The solu-

tion contained 0.01% sodium azide.

## 3 Results

### 3.1 Immunohistochemistry and confocal laser microscopy

Nectin-2 was expressed around the heads of maturing spermatids, as well as the basal part of the seminiferous epithelium (Figure 1A). The localization around the heads corresponded to the ectoplasmic specialization between the Sertoli cells and maturing spermatids; the localization at the basal part corresponded to the ectoplasmic specialization between adjoining Sertoli cells. In the negative control sections, the signal for nectin-2 was not detected (Figure 1B).

Nectin-3 was expressed around the heads of maturing spermatids (Figure 2A), corresponding to the ectoplasmic specialization between the Sertoli cells and ma-

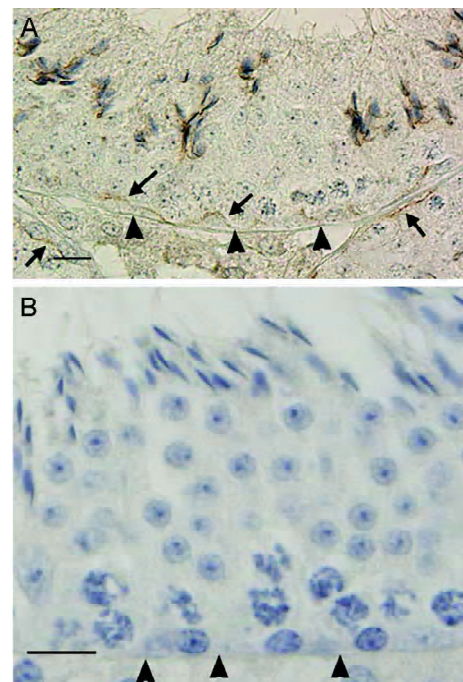


Figure 1. Seminiferous epithelium of mouse testis stained with anti-nectin-2 antibody (3,3'-diaminobenzidine reaction) (A) and the negative control (B). Nectin-2 is localized around the heads of elongated spermatids, corresponding to the Sertoli-maturing spermatid ectoplasmic specialization. Nectin-2 is also localized at the ectoplasmic specialization between adjoining Sertoli cells. Arrows show examples. The control section shows no reactivities. Arrowheads show the basement membrane including myoid cells. Hematoxylin was used as the counterstain. Bars = 20  $\mu$ m.

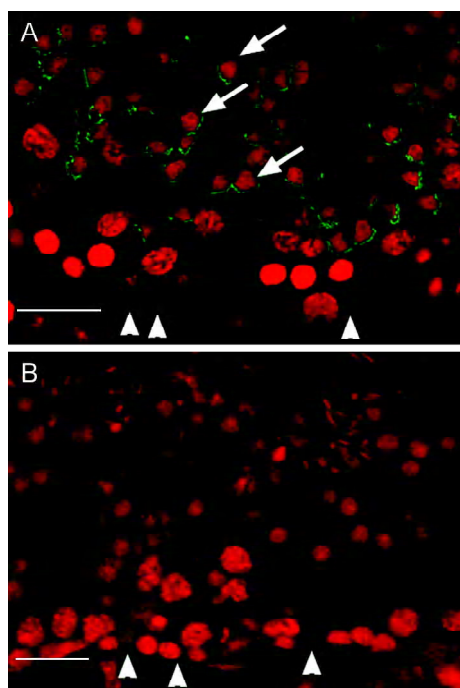


Figure 2. Seminiferous epithelium of mouse testis stained with anti-nectin-3 antibody (A) and the negative control (B). Nectin-3 is localized around the heads of elongating spermatids (green in color), corresponding to the Sertoli-maturing spermatid ectoplasmic specializations. Examples are indicated by arrows. Step 9 spermatids are shown. Nectin-3 is not expressed at the ectoplasmic specialization between adjoining Sertoli cells. There is no staining in the control section. Arrowheads show the basement membrane including myoid cells. Propidium iodide was used as the counterstain (red in color). Bars = 20  $\mu$ m.

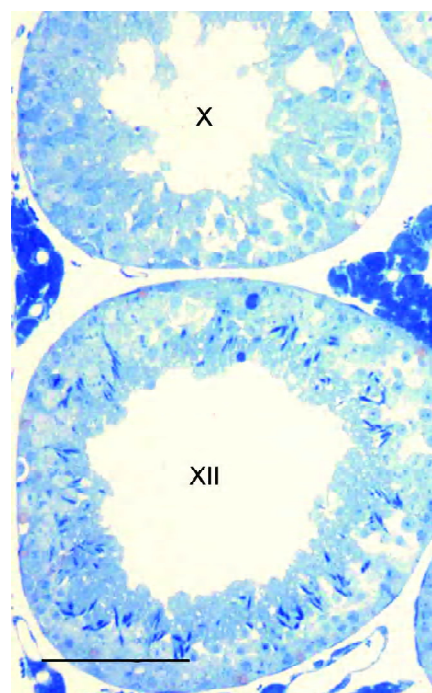


Figure 3. Seminiferous tubules of mouse testis micro-injected with the control solution containing 0.01% sodium azide, stained with toluidine blue. Stage X and XII epithelia are shown. Note enlarged intercellular spaces in the seminiferous epithelium. Bar = 100  $\mu$ m.

turing spermatids. Nectin-3 was not expressed at the ectoplasmic specialization between the adjoining Sertoli cells (Figure 2A). The negative control sections showed no reactivities (Figure 2B).

### 3.2 Micro-injection experiments

The seminiferous tubules micro-injected with the solution without antibody (negative control experiments) showed minor ultrastructural changes, such as the presence of enlarged vacuoles in the Sertoli cells and in the spermatocytes. The intercellular space tended to be enlarged (Figure 3). The ectoplasmic specialization between the Sertoli cells and the maturing spermatids in the control experiments was intact together with the actin layers and subsurface cistern of the endoplasmic reticulum (Figure 4). The ectoplasmic specialization between adjoining Sertoli cells was also intact.

Five hours after micro-injection of anti-nectin-2 antibody, the histological sections of the seminiferous tubules showed similar features to the control testis, except for the exfoliation of maturing spermatids (Figure 5). Electron microscopy at this stage showed that part

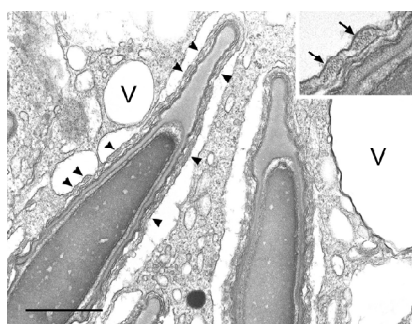


Figure 4. Electron micrograph of mouse elongated spermatids. The seminiferous tubule was micro-injected with the control solution containing 0.01% sodium azide. The testis was fixed 12 h after injection. The head of the spermatid is normal in structure. Arrowheads show an ectoplasmic specialization between a Sertoli cell and the spermatid. Bundles of actin filaments at the specialization are clearly seen in the inset (arrows). Enlarged vacuoles (V) in the Sertoli cell are observable. Bar = 1  $\mu$ m.

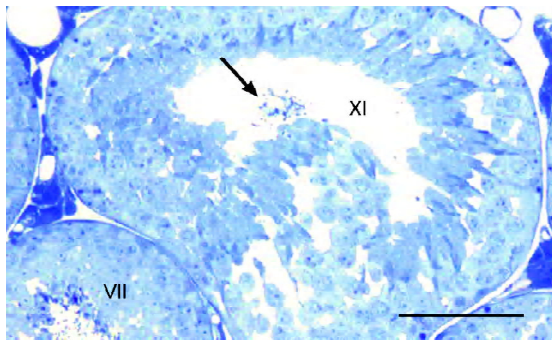


Figure 5. A stage XI seminiferous tubule of mouse testis micro-injected with anti-nectin-2 antibody. The testis was fixed 5 h after injection. A cluster of mature spermatids (arrow) is seen. As they are not step 11 spermatids, they were exfoliated from nearby epithelium. Enlarged intercellular spaces and, at the upper left, a stage VII tubule with normal configuration can be observed. Anti-nectin-2 antibody seems not to have penetrated this tubule. Note that many step 16 spermatids can be seen in the adluminal compartment. Bar = 100  $\mu$ m.

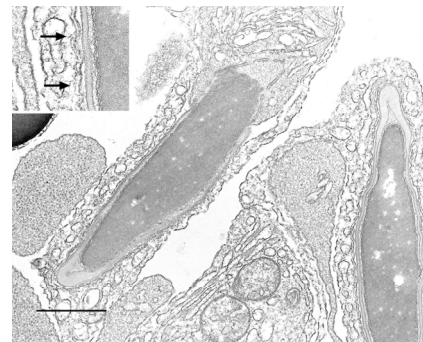


Figure 7. Electron micrograph of mouse elongated spermatids. The seminiferous tubule was fixed 12 h after micro-injection of anti-nectin-2 antibody. Note no ectoplasmic specializations can be observed over the acrosomes. The inset shows part of the right spermatid. No specialization is observable (arrows in the inset). Bar = 1  $\mu$ m.

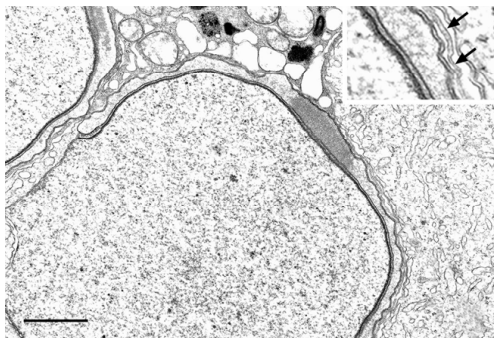


Figure 6. Electron micrograph of a mouse step 8 spermatid. The testis was fixed 12 h after micro-injection of anti-nectin-2 antibody. Arrowheads show the acrosomal cap. Note no ectoplasmic specialization can be observed over the acrosomal cap. Arrows in the inset show an attenuated process of the Sertoli cell. Bar = 1  $\mu$ m.

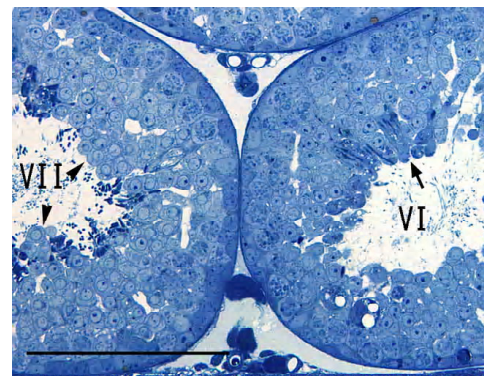


Figure 8. Seminiferous tubules of mouse testis micro-injected with anti-nectin-3 antibody. The testis was fixed 5 h after injection. Stage VI and VII tubules are shown. In the right tubule, most step 16 spermatids have been exfoliated from the epithelium. Some spermatids are still in the epithelium (arrows). In the left tubule, some spermatids have been exfoliated (arrowheads). Bar = 100  $\mu$ m.

of the actin layer in the ectoplasmic specialization between the Sertoli cells and maturing spermatids was disrupted. By 12 h, the actin layer had completely disappeared (Figures 6, 7), and maturing spermatids were observed in the lumen of the seminiferous tubules because of detachment. The ectoplasmic specialization covering the acrosomal cap of a step 8 spermatid was also affected by the anti-nectin-2 antibody (Figure 6).

Micro-injection of anti-nectin-3 antibody showed

similar structural changes. By 5 h after the injection, maturing spermatids were exfoliated (Figure 8), and by 12 h after injection the actin layer at the ectoplasmic specialization between Sertoli cells and spermatids had completely disappeared (Figures 9, 10).

After micro-injection of anti-afadin antibody, no drastic changes in the ultrastructure were observed. The ultrastructure was similar to that in the negative control animals.



Figure 9. Mouse elongated spermatids are shown with seminiferous tubules fixed 12 h after micro-injection of anti-nectin-3 antibody. The spermatids were about to exfoliate. As a step 7 spermatid can be observed on the right-hand side of the figure, the stage of the seminiferous tubule is VII. Note no ectoplasmic specializations can be observed over the acrosomes. The inset shows a higher magnification of part of the right spermatid. Note no actin filaments are observable (arrows in the inset). Bar = 1  $\mu$ m.



Figure 10. Mouse elongated spermatids with seminiferous tubule fixed 12 h after micro-injection of anti-nectin-3 antibody. Note no ectoplasmic specializations can be observed. The inset shows part of the middle spermatid at higher magnification. No actin filaments are observable (arrows in the inset). Bar = 1  $\mu$ m.

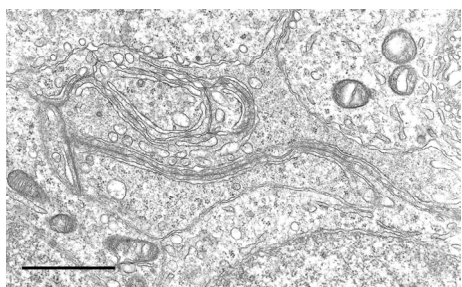


Figure 11. Sertoli-Sertoli ectoplasmic specialization observed in a seminiferous tubule of mouse testis micro-injected with anti-nectin-2 antibody. The testis was fixed 12 h after injection. The ultrastructure of the specialization is normal. Bar = 1  $\mu$ m.

The Sertoli-Sertoli ectoplasmic specialization was not affected by either anti-nectin-2, anti-nectin-3, or anti-afadin antibodies. The specialization showed an intact ultrastructure with actin filaments and the subsurface cistern (Figure 11).

#### 4 Discussion

The antibody solutions used in the present study were micro-injected into the lumen of seminiferous tubules. The solution filled the lumen, penetrated into the intercellular space in the adluminal compartment of the seminiferous epithelium and stopped penetrating at the adluminal edge of the ectoplasmic specialization between Sertoli cells. The solution also penetrated into the intercellular space at the ectoplasmic specialization between Sertoli cells and maturing spermatids. This was already shown by Toyama *et al.* [15], who micro-injected cytochrome c into the lumen of the seminiferous tubules of mouse and rat and electron microscopically traced the intercellular space after a 3,3'-diaminobenzidine reaction.

Nectins have carboxyl termini in the cytoplasmic domain and N-termini in the extracellular domain. As the anti-nectin-2 antibody used in the present study was raised against the amino acid residues near the N-terminus, the epitope was exposed in the intercellular space at the Sertoli cell-maturing spermatid ectoplasmic specialization and the antibody bound the epitope of nectin-2. As a result, nectin-2 generated a conformational change and appeared to disrupt the actin filaments in the Sertoli cells through the nectin-afadin-actin system.

As disruption of the actin filaments at the ectoplasmic specialization between Sertoli cells and maturing spermatids was not observed in the negative control experiments, the disruption was a result of the injection of the anti-nectin-2 antibody. Both the antibody mixture and the control mixture contained 0.01% sodium azide, therefore the disruption was not an adverse effect of sodium azide.

Micro-injected anti-nectin-3 antibody, as well as anti-nectin-2 antibody, penetrated into the intercellular space in the adluminal compartment. The anti-nectin-3 antibody used in the present study was also raised against the recombinant fragment, corresponding to the mouse nectin-3 extracellular domain. The seminiferous tubules micro-injected with anti-nectin-3 antibody showed similar adverse effects, including disruption of the actin filaments in Sertoli cells at the Sertoli-maturing spermatid

ectoplasmic specialization and exfoliation of maturing spermatids from the seminiferous epithelium. The presence of afadin and F-actin in the spermatid at the specialization has not yet been ascertained. The results of the present study suggest that nectin-3 in the spermatids affected the actin filaments in the Sertoli cells through nectin-2 and afadin. A possible but highly speculative explanation to these adverse effects is as follows. Anti-nectin-3 antibody bound the epitope of nectin-3, which is exposed in the intercellular space at the specialization, and changed nectin-3, which in turn affected nectin-2, which was forming the hetero-*trans*-dimer with nectin-3. Nectin-2, affected by the inactivated nectin-3 in the spermatids, seemed to disrupt the actin filaments in the Sertoli cells through the nectin-afadin-actin system, as mentioned above. Nectin-3 and nectin-2 finally disrupted the actin filaments at the ectoplasmic specialization between Sertoli cells and maturing spermatids. This is supported by the fact that nectin-2 at the ectoplasmic specialization between Sertoli cells and maturing spermatids completely disappeared in nectin-3 knockout mice [11]. The close relationship between nectin-2 and nectin-3 was also reported in nectin-2 and nectin-3 knockout mice [8–11]. Male infertility, because of malformed spermatozoa, was the prominent feature. The heads of spermatozoa from both nectin-2 and nectin-3 knockout mice were heterogeneous in shape and had malformed acrosomes and large vacuoles. The tails were coiled up around the heads and the mitochondrial sheaths were disorganized. The mitochondria were detached from the outer dense fibers in the middle piece and came together in the heads, forming the so-called “stratified mitochondrial sheaths”. Similar abnormalities were observed in spermatozoa from Golgi-associated PDZ- and coiled-coil motif-containing protein-deficient mice [16]. The state of the spermatozoa from these mice is known as a globozoospermia, lacking acrosomes and posterior rings in their heads.

The seminiferous tubules micro-injected with anti-afadin antibody showed no remarkable changes, as expected. As afadin has neither a transmembrane domain nor an extracellular domain, it is reasonable that the antibody, micro-injected into the intercellular space at the Sertoli-maturing spermatid specialization, did not bind any epitope in afadin.

Vacuoles in the Sertoli cells and enlarged intercellular spaces were observed in all experimental systems (Figures 5–10), including the negative controls (Figures 3, 4). As the solution for all experimental systems con-

tained 0.01% sodium azide, it might cause the enlarged vacuoles and intercellular spaces.

The present study suggests that the ectoplasmic specialization between Sertoli cells and maturing spermatids holds the spermatids until spermiation, as was put forward by Brokelmann [17], Nicander [18], and Fawcett [19]. Similar results, deletion of the ectoplasmic specialization between Sertoli cells and maturing spermatids, were reported in mouse testes treated with estrogen or estrogen-like chemicals, such as bisphenol A [15, 20]. The researchers also concluded that the ectoplasmic specialization shapes the nucleus of the mature spermatids, as the chemicals induced malformation of the heads of mature spermatids.

Injected antibodies directly bind and inactivate the proteins that are expressed at the ectoplasmic specialization between Sertoli cells and maturing spermatids, therefore this experimental system, a micro-injection system, as well as the generation of knockout mice, are useful tools for understanding the function of the specialization. However, one disadvantage of this experimental system is that antibody concentration in the lumen cannot be controlled.

The ectoplasmic specialization between adjoining Sertoli cells equipped with tight junctions was not affected by the antibodies used (anti-nectin-2, anti-nectin-3, and anti-afadin antibodies). Because the antibody did not bind the antigen, the conformational change in the antigen (nectin-2, nectin-3, and afadin) did not occur. As a result, the ultrastructure and function of the specialization remained intact. Explanations for this are as follows. As anti-nectin-2 antibody micro-injected into the lumen of the seminiferous tubule penetrated into the intercellular spaces in the adluminal compartment of the seminiferous epithelium and was stopped at the uppermost strand of the tight junction, just like cytochrome c micro-injected into the lumen [15], the antibody did not reach the epitope. The anti-nectin-3 antibody was not localized at the specialization, as shown in Figure 2A. Because afadin has neither a transmembrane domain nor an extracellular domain, the antibody did not bind the epitope.

The function of the blood-testis barrier was also intact. This is supported by the fact that normal spermatogenesis, except for detachment of maturing spermatids, was observed. Without the blood-testis barrier, spermatogenesis is arrested at meiosis. Although spermatozoa were malformed and infertile, nectin-2 and nectin-3 knockout mice showed spermatogenesis [8–11], suggest-

ing that the function of the blood–testis barrier in these mice was normal.

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