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A testis-specific serine protease, Prss41/Tessp-1, is necessary for the progression of meiosis during murine *in vitro* spermatogenesis

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ABSTRACT

The function of protease during male meiosis has not been well studied. We previously cloned and characterized four testis-specific serine proteases in the mouse testis. One of the proteases, Prss41/Tessp-1, was expressed in the germ and Sertoli cell. This time, to examine the involvement of Prss41/Tessp-1 in spermatogenesis, we conducted the organ culture of testis fragments in the presence of the anti-Prss41/Tessp-1 antibody. Because in the Sertoli cell, the Prss41/Tessp-1 protein was mostly associated with the membrane of intracellular organelles by glycosylphosphatidylinositol, the antibody was expected to affect Prss41/Tessp-1 at the plasma membrane of spermatogonia. By adding the antibody, the number of germ cells was decreased in some seminiferous tubules. The marker genes expression strongly suggested that meiosis was arrested at spermatogonia, and the number of apoptotic germ cells increased by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. These data indicated that Prss41/Tessp-1 was necessary for the progression of meiosis at the stage of spermatogonia during *in vitro* spermatogenesis. Together with our previous study, the current results suggest that the Prss/Tessp proteases are important for the progression of meiosis at each stage.

1. Introduction

Proteases are known to play important roles in many physiological events [1,2]. However, there are about 500 protease-related genes in mammals [3], and the function of protease still needs to be elucidated. We have focused on serine proteases, which are the largest family among the four protease families in mammals [3], during meiosis in murine spermatogenesis. We cloned four testis-specific serine proteases from the mouse testis, and three of them, namely, Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4, are encoded by genes that consist of a cluster at chromosome 9 [4,5]. Although the three genes are paralogs that have similar primary structures, their functions seem to be different. Especially, Prss42/Tessp-2 and Prss43/Tessp-3 are necessary for the progression of meiosis in secondary and primary spermatocytes, respectively, during *in vitro* spermatogenesis [5].

The gene encoding Prss41/Tessp-1 is located at mouse chromosome 17 and forms another gene cluster with other serine proteases [6]. Prss41/Tessp-1 mRNA is expressed in spermatogonia and spermatocytes as well as Sertoli cells in the mouse testis [4,7]. At the protein level, Prss41/Tessp-1 is localized at the plasma membrane of spermatogonia and in the Golgi apparatus of spermatocytes and spermatids [7], although its localization in the Sertoli cell is unclear. By overexpression in the COS-7 cell, the Prss41/Tessp-1 protease is presumed to be a glycosylphosphatidylinositol (GPI)-anchored protein [4]. However, there are currently no data indicating or suggesting the function of Prss41/Tessp-1 during spermatogenesis.

In this study, we conducted the organ culture of testis fragments to examine the function of Prss41/Tessp-1 in spermatogenesis. By culturing testis fragments with the antibody against Prss41/Tessp-1, meiosis was arrested at the stage of spermatogonia and the number of apoptotic cells increased. The results suggest that Prss41/Tessp-1 has a role during meiosis, especially when spermatogonia differentiate into primary spermatocytes.

2. Materials and methods

2.1. Animals

C57/BL6 mice were maintained at 25 °C with a photoperiod of 14:10 (light:dark) and with free access to food and water. Experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

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2.2. Isolation of germ cells and their fractionation into nuclear, membrane, and cytoplasmic subfractions

Germ cells were isolated from the mouse testis as previously described [5]. The germ cells were further fractionated into nuclear, membrane, and cytoplasmic subfractions as previously described [8].

2.3. Primary culture of Sertoli cells

Testes were obtained from mice at 7–12 days after birth. After the tunica albuginea was removed, the tissues were placed in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan) and incubated at 32 °C for 20 min with occasional inversion. The seminiferous tubules were separated from dispersed interstitial cells by unit gravity sedimentation for 5 min, and the supernatant was discarded. The tubules were placed again in DMEM containing 0.1% collagenase and incubated at 32 °C for 15 min with occasional inversion. The tubules were collected by unit gravity sedimentation for 5 min and washed with phosphate buffered saline (PBS) containing 1 mM EDTA for three times. Then, the tissues were cut into small pieces by scissors, and we pipetted them with a 1-ml bored tip several times and dispersed Sertoli and germ cells in PBS containing 1 mM EDTA. The dispersed cells were separated from tissue debris by unit gravity sedimentation for 5 min and the supernatant was collected as the Sertoli cell fraction with germ cell contamination. The cells were then precipitated by the centrifugation at 70×*g* for 5 min at room temperature and washed with DMEM/F12 containing 10% fetal bovine serum (FBS) three times. The cells were suspended in DMED/F12 containing 10% FBS and spread onto 24-well plates or 10-cm dishes. After the culture for 24 h at 32 °C, we discarded the medium, washed the cells with PBS, and treated them with 10 mM Tris–HCl (pH 7.4) for 3 min at room temperature to eliminate germ cells. The floated cells were discarded, and the attached cells were washed with PBS and the new medium was added. Generally, we cultured the Sertoli cells at 32 °C for 5–6 days before use.

2.4. Western blot analysis

Western blot analysis was conducted as previously described [5]. The antibody against Prss41/Tessp-1 was generated before [7]. To confirm signal specificity, we also used the antibody that was pre-incubated with the Prss41/Tessp-1 antigen.

2.5. Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Germ cells or the membrane fraction of Sertoli cells were treated with or without PI-PLC (Sigma, St. Louis, MO, 0.2 U/ml) as previously described [4,5].

2.6. Immunocytochemistry of the Sertoli cell

Sertoli cells were cultured in a 24-well plate and were fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan). After being washed with PBS for three times, the cells were treated with 3% hydrogen peroxide (Wako Pure Chemicals) and washed again with PBS. The cells were then blocked with Block Ace (Dainippon-Sumitomo seiyaku) for one hour and incubated with the purified Prss41/Tessp-1 antibody (10 μg/ml) diluted with PBS containing 0.1% Tween 20 (TPBS). After one hour of incubation at room temperature, the cells were washed with TPBS and incubated with donkey anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare Biosciences) diluted with TPBS at a ratio of 1:500 for 1 h at room temperature. After washing with TPBS, signals were detected using an AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) at room temperature.

2.7. Organ culture of testis fragments with the Prss41/Tessp-1 antibody

Organ culture of mouse testis fragments was performed as previously described [5], but this time we harvested 7-day-old testes and cultured for 14 days. A part of cultured testis fragments were fixed with Bouin's solution for 18 h at 4 °C, and paraffin sections were made to be stained with hematoxylin. Another part of the tissue was used for total RNA isolation and subject to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for the expression of marker genes at different meiotic stages [5].

2.8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was conducted as previously described [5]. Briefly, paraffin sections were prepared after the organ culture and the apoptotic cells were detected by *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. The slides were counterstained with Hoechst 33342 and observed under a fluorescence microscope.

2.9. Statistical analysis

Results are given as mean ± S.D. Statistical comparisons were made by Student's *t*-test.

3. Results

3.1. The localization of Prss41/Tessp-1 in native testicular germ cells and Sertoli cells

In our previous study, we showed that the Prss41/Tessp-1 protein was localized at the plasma membrane of spermatogonia and in the Golgi apparatus of later stages of germ cells [7]. We also found that the Sertoli cell expressed Prss41/Tessp-1, but its subcellular localization was not clear. Therefore, in this study, we first investigated the subcellular distribution of Prss41/Tessp-1 in isolated germ cells and cultured Sertoli cells. We fractionated the germ cell and the Sertoli cell into nuclear, membrane, and cytoplasmic subfractions and conducted western blot analysis. As a result, Prss41/Tessp-1 was detected specifically in the membrane fraction in both the germ cell and the Sertoli cell (Fig. 1A).

Prss41/Tessp-1 is supposed to be a GPI-anchored protein. However, this is based on the localization in the COS-7 cell overexpressing the Prss41/Tessp-1 protein [4], so we next examined if this protease is a GPI-anchored protein in isolated germ cells and cultured Sertoli cells. We treated the intact germ cell or the membrane fraction of the Sertoli cell with PI-PLC and examined if the protein was released from the cell membrane. Urokinsase-type plasminogen activator (uPAR) was used as a positive control to confirm that we successfully digested a GPI-anchored protein. We detected a specific signal for Prss41/Tessp-1 in the supernatant after the treatment in both germ and Sertoli cells, but no signal was observed without the enzyme (Fig. 1B). This indicated that Prss41/Tessp-1 was a GPI-anchored membrane protein in native testicular germ cells and Sertoli cells.

To see if Prss41/Tessp-1 was linked to the plasma membrane or intracellular compartments in the Sertoli cell, we conducted immunocytochemistry of the cultured Sertoli cell. The specific signal was detected inside the cell, especially in the perinuclear region, but not in the nucleus (Fig. 1C). This was consistent with the fact that, in our PI-PLC assay, we failed to detect any

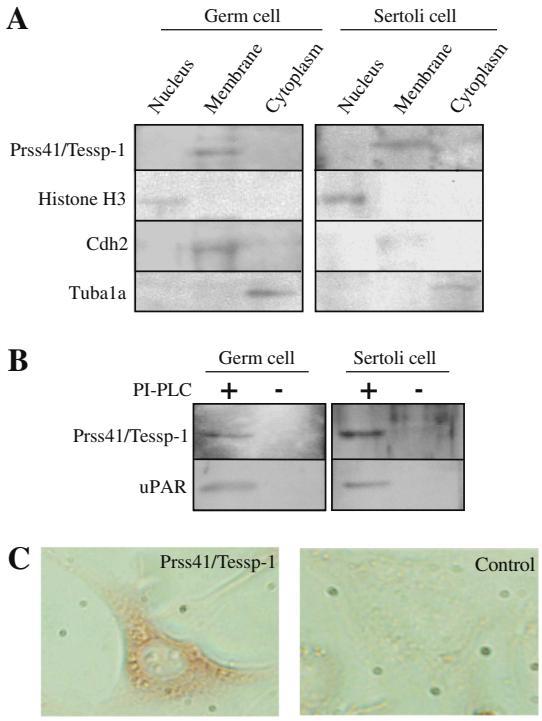

Fig. 1. Subcellular localization of the Prss41/Tessp-1 protein. (A) Western blot analysis with the nuclear, membrane, and cytoplasmic subfractions of the mouse testicular germ cell and the Sertoli cell. Native testicular germ cells were isolated from adult male mice. Sertoli cells were purified from 7 to 12-day-old mouse testes and cultured for 5–6 days before the experiment. The cells were further fractionated into each subfraction and used for western blot. Histone H3, Cdh2, and Tuba1a were detected as markers for the nuclear, membrane, and cytoplasmic subfractions, respectively. (B) Western blot analysis after PI-PLC treatment. The intact germ cell or the membrane fraction from the Sertoli cell was treated with (+) or without PI-PLC (−) and the supernatant was used for western blot analysis with the Prss41/Tessp-1 antibody. uPAR was used as a positive control. (C) Immunocytochemistry of the Sertoli cell. Sertoli cells were cultured in the 24-well plate and subjected to immunostaining with the Prss41/Tessp-1 antibody (Prss41/Tessp-1) or the antibody preabsorbed with the Prss41/Tessp-1 antigen (control).

Prss41/Tessp-1 signal by treating the intact Sertoli cell with the enzyme (data not shown). Although we could not completely rule out the possibility that the protein was also localized in the plasma membrane, these results strongly suggested that Prss41/Tessp-1 was mostly associated with intracellular organelles in the Sertoli cell.

3.2. Organ culture with the Prss41/Tessp-1 antibody results in meiotic arrest of spermatogonia

To examine the role of Prss41/Tessp-1 in spermatogenesis, we conducted the organ culture of testis fragments in the presence of the specific Prss41/Tessp-1 antibody. We previously performed this experiment to investigate the functions of Prss42/Tessp-2 and Prss43/Tessp-3 [5]. At that time, we isolated testis fragments from 2-week-old mice because these proteases began to be expressed at the primary spermatocyte stage [5]. In contrast, Prss41/Tessp-1 began to be produced as early as at the stage of spermatogonia, so this time, we harvested 7-day-old testes for the organ culture. At this age, the testis mostly contained spermatogonia and Sertoli cells in the seminiferous tubule [9].

We cultured the fragments for two weeks with the Prss41/Tessp-1 antibody, the antibody preincubated with the Prss41/Tessp-1 antigen, or the antibody against medaka MT2-MMP [10] which was not produced in the mouse testis. After the culture, we made a paraffin section for each sample and observed it to see if spermatogenesis was affected. By the culture with the preabsorbed antibody or with the medaka MT2-MMP antibody, all the seminiferous tubules contained many cells (Fig. 2A, B). In contrast, when we cultured the tissue with the Prss41/Tessp-1 antibody, some seminiferous tubules contained very few cells (Fig. 2D), although there were other tubules in which many cells were observed (Fig. 2C). This suggested that meiosis was impaired in some tubules by adding the Prss41/Tessp-1 antibody.

To determine what kinds of germ cells were included in the cultured tissues, we examined the expression of marker genes for meiotic stages by qRT-PCR. We used *rnh2* (NLR family, pyrin domain containing 4C), *sycp2* (synaptonemal complex protein 2), *pten2* (transmembrane phosphatase with tensin homology), and *prm1* (protamine 1) as marker genes for spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, respectively [5]. While the expression of all the marker genes was similar in the tissue cultured with the MT2-MMP anti-

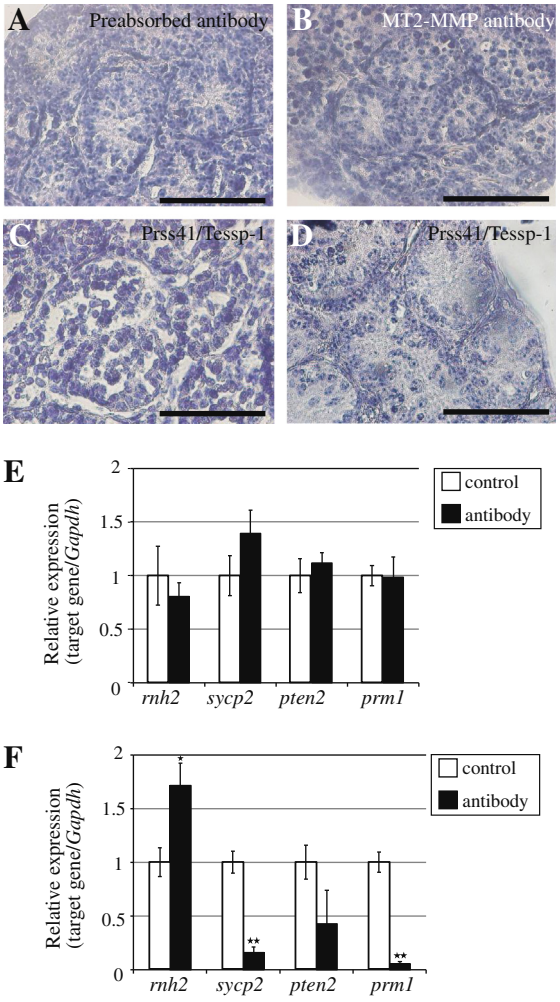

Fig. 2. Organ culture of testis fragments with the Prss41/Tessp-1 antibody. (A–D) Testis fragments were prepared from the 7-day-old mice and cultured with the Prss41/Tessp-1 antibody (C, D). As controls, the fragments were also cultured with the antibody preabsorbed with the Prss41/Tessp-1 antigen (A) and the medaka MT2-MMP antibody (B). The paraffin sections were stained with hematoxylin and eosin. Scale bars = 100 μm. (E, F) The marker genes expression in the testis fragments cultured with the medaka MT2-MMP antibody (E) or with the Prss41/Tessp-1 antibody (F). The expression of marker genes for each meiotic stage was examined by qRT-PCR. The relative expression level is shown as fold difference calculated relative to the expression in the control tissues that were cultured with the preabsorbed antibody. Values are means ± S.D. ($^{*}p < 0.05$; $^{**}p < 0.01$ compared with control, $n = 3$).

body to the control (Fig. 2E), it was changed by adding the Prss41/Tessp-1 antibody. The *rnh2* expression significantly elevated by 1.7-fold compared to the control, and the expression of *sycp2* and *prm1* was dramatically decreased (Fig. 2F). The *pten2* mRNA level was also decreased, but the difference from the control was not statistically significant. The data indicated that the addition of the Prss41/Tessp-1 antibody increased the population of spermatogonia and decreased the germ cell at later stages than that. This strongly suggested that the antibody arrested meiosis at the stage of spermatogonia.

3.3. The addition of the Prss41/Tessp-1 antibody increased the number of apoptotic cells

We finally assessed whether the germ cells underwent apoptosis when the testis fragments were cultured with the Prss41/Tessp-1 antibody by TUNEL assay. The tissue showed many more apoptotic cells by adding the Prss41/Tessp-1 antibody compared to the testis fragments cultured with the control antibodies (Fig. 3A). The TUNEL-positive signals appeared to be observed mainly from cells inside the seminiferous tubules, which suggested that many cells undergoing apoptosis might be germ cells. This was supported by the qRT-PCR data showing that by adding the Prss41/Tessp-1 antibody, the marker gene expression for germ cells (*Ddx4*) decreased but the markers for the Sertoli and Leydig cell (*Clu* and *Hsd3b*) was unchanged (Fig. 3B). Therefore, we concluded that the addition of the Prss41/Tessp-1 antibody led germ cells to apoptosis during *in vitro* spermatogenesis.

4. Discussion

We first investigated the subcellular localization of Prss41/Tessp-1 protein in the Sertoli cell and found that it was a GPI-anchored protein associated with certain intracellular organelles (Fig. 1). By immunocytochemistry, most signals for Prss41/Tessp-1 were observed in the cytosol although a few signals might also be present on the plasma membrane. It is hard to tell which intracellular organelle was associated with Prss41/Tessp-1, but this result strongly suggests that the Prss41/Tessp-1 protease performs its function inside the Sertoli cell. Together with our previous results showing that Prss41/Tessp-1 was localized at the plasma membrane of spermatogonia and in the Golgi apparatus of spermatocytes and spermatids [7], the Prss41/Tessp-1 antibody was likely to affect the protein produced in spermatogonia during our organ culture.

The organ culture with the Prss41/Tessp-1 antibody resulted in the increase of spermatogonia and the decrease of later stages of germ cells. This indicated that meiosis stopped at the stage of spermatogonia. However, the fold increase in spermatogonia was only 1.7, which was in contrast to several folds in spermatocytes when

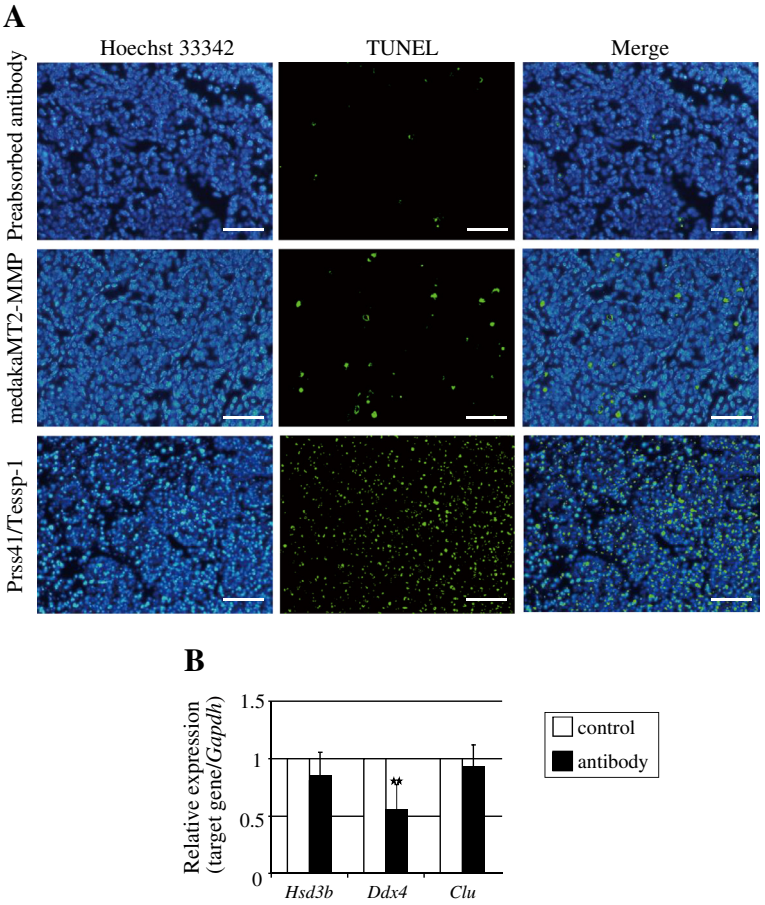

Fig. 3. Increased apoptosis in the testis fragments cultured with the Prss41/Tessp-1 antibody. (A) TUNEL assays of the testis fragments cultured with the preabsorbed Prss41/Tessp-1 antibody (top), the medaka MT2-MMP antibody (middle), and the Prss41/Tessp-1 antibody (bottom). Testis fragments obtained from 7-day-old mice were cultured with the indicated antibodies for 14 days. Paraffin sections were prepared from the cultured fragments, and the cells undergoing apoptosis were labeled using TdT and fluorescein-dUTP (green). Cell nuclei were counterstained with Hoechst 33342 (blue). Scale bars = 50 μm. (B) The expression of marker genes of Leydig, germ and Sertoli cells. The expression of each marker gene was examined by qRT-PCR. *Hsd3b*, *Ddx4*, and *Clu* were used as marker genes for Leydig, germ, and Sertoli cells, respectively. Values are means ± S.D. ($^{*}p < 0.05$ compared with control, $n = 3$). (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

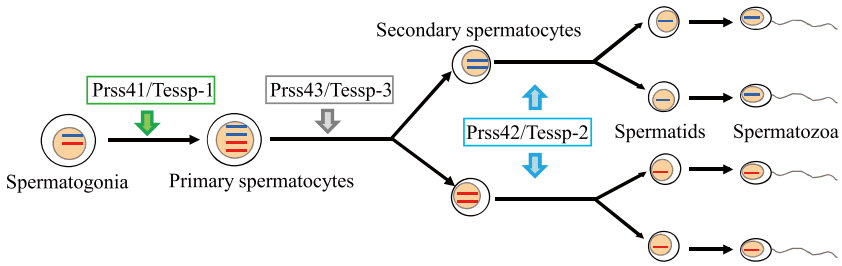

Fig. 4. The involvement of the Prss/Tessp proteases at each step of meiosis. According to the data of our organ culture with each Prss/Tessp antibody, the Prss/Tessp proteases are necessary for the progression of meiosis. Prss41/Tessp-1 is needed for spermatogonia to differentiate into primary spermatocytes. Prss43/Tessp-3 is necessary for the differentiation from primary spermatocytes to secondary spermatocytes, and Prss42/Tessp-2 from secondary spermatocytes to spermatids.

we cultured the testis fragments with the antibodies against Prss42/Tessp-2 or Prss43/Tessp-3 [5]. This was probably because Prss41/Tessp-1 was expressed in type B spermatogonia but not in type A spermatogonia [4]. While the Prss41/Tessp-1 antibody could only affect type B spermatogonia, type A spermatogonia could self-propagate and increase their number in both the experiment with the Prss41/Tessp-1 antibody and the control experiment. The marker gene we used, *rnh2*, was expressed in both types of spermatogonia [11], so we could only observe a slight increase.

Although the levels of marker genes for spermatocytes and spermatids decreased by adding the Prss41/Tessp-1 antibody, the decrease of the *pten2* expression in secondary spermatocytes was not statistically significant. This was possibly because some primary spermatocytes might exist when we started the organ culture. Since Prss41/Tessp-1 was localized to the Golgi apparatus in primary spermatocytes, the protein was not influenced by the Prss41/Tessp-1 antibody in these cells and meiosis could continue. However, two weeks from 7 days after birth might not be enough for the germ cells to reach the spermatid stage, so most of them might be secondary spermatocytes. Indeed, the 3-week-old testis was reported to contain only a few spermatids [9].

Alternatively, the formation of the blood-testis-barrier (BTB) might be related to this. Generally, BTB is formed at 10 days after birth in the mouse testis [12], and it physically separates the seminiferous epithelium into basal and apical parts and functionally works for an immune defense [13,14]. When we started the organ culture from 7-day-old testes, BTB should not yet be formed. If BTB was completed in the middle of our organ culture, it might interfere with the access of the Prss41/Tessp-1 antibody. This might allow more germ cells to undergo normal meiosis and resulted in the increase of secondary spermatocytes even in the presence of the Prss41/tessp-1 antibody.

The addition of the antibody led germ cells, possibly type B spermatogonia, to apoptosis. As we previously discussed, meiotic arrest could cause germ cell apoptosis [5]. In case of this study, the antibody probably bound to the Prss41/Tessp-1 protein on the plasma membrane of type B spermatogonia and blocked its function. This inhibition was likely to cause meiotic arrest of the germ cells, and eventually their apoptosis. As we observed by our TUNEL assay, most seminiferous tubules contained apoptotic cells regardless of the cell number in them (Fig. 3). We presume that type B spermatogonia in most tubules might undergo apoptosis but the timing of their removal might be different. This might resulted in the difference of the cell number between seminiferous tubules. In any case, the present data strongly suggested that Prss41/Tessp-1 in spermatogonia was necessary for the progression of meiosis at least in our *in vitro* spermatogenesis system.

How is Prss41/Tessp-1 involved in meiosis? Because this protein is a protease, we assume that Prss41/Tessp-1 might degrade the junction between the germ and Sertoli cell. Especially, it is very important for germ cells to go through BTB and migrate into the inner lumen of seminiferous tubules. BTB is composed of several protein complexes, and for its dynamics, many proteins such as growth factors, proteases, and protease inhibitors are thought to be important [13,14]. Generally, the germ cell passing through BTB is at the stage of preleptotene spermatocytes [15]. Considering that the preleptotene stage comes just after type B spermatogonia and that we have not determined which types of primary spermatocytes actually contained Prss41/Tessp-1 in the Golgi apparatus, it may be possible that this protease is present at the plasma membrane of preleptotene spermatocytes and plays a role in its migration through BTB.

Collectively, the current data suggest that Prss41/Tessp-1 is necessary for the progression of meiosis at the stages from spermatogonia to primary spermatocytes. This is interesting because we have already reported that other testis-specific serine proteases are necessary for the progression of meiosis at different stages [5]. Prss43/Tessp-3 is necessary for primary spermatocytes to differentiate into secondary spermatocytes, and Prss42/Tessp-2 is needed in the process from secondary spermatocytes to spermatids. These data propose that the Prss/Tessp proteases in the plasma membrane of differentiating germ cells are essential for the progression of each step of meiosis (Fig. 4). Further investigation is necessary for understanding how these proteases are involved in the male meiosis.

In summary, Prss41/Tessp-1 was a GPI-anchored protein in both the germ cell and the Sertoli cell. Adding the Prss41/Tessp-1 antibody to the organ culture of testis fragments arrested meiosis in spermatogonia and induced germ cell apoptosis. Together with our previous data, Prss/Tessp proteases probably play important roles in spermatogenesis.

Disclosure summary

The authors certify that they have nothing to disclose.

Acknowledgments

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