Three testis-specific paralogous serine proteases play different roles in murine spermatogenesis and are involved in germ cell survival during meiosis[1]

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Running title: A role of serine protease in spermatogenesis

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Summary sentence: Three testis-specific paralogous Prss/Tessp proteases are expressed differently during murine spermatogenesis, and two of these are required for germ cell survival in *in vitro* spermatogenesis.

Key words: Protease; Prss; Tessp; germ cell; localization; meiosis; spermatogenesis; organ culture; apoptosis

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ABSTRACT

Spermatogenesis is a complex process that generates spermatozoa; its molecular mechanisms are not completely understood. Here we focused on the functions of three testis-specific serine proteases: Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4. These protease genes, which constitute a gene cluster on chromosome 9F2-F3, were presumed to be paralogs and were expressed only in the testis. By investigating their mRNA distribution, we found that all three genes were expressed in primary and secondary spermatocytes. However, interestingly, the translated proteins were produced at different locations. Prss42/Tessp-2 was found in the membranes and cytoplasm of secondary spermatocytes and spermatids, whereas Prss43/Tessp-3 was present only in the membranes of spermatocytes and spermatids. Prss44/Tessp-4 was detected in the cytoplasm of spermatocytes and spermatids. To assess the roles of these proteases in spermatogenesis, we used organ culture of mouse testis fragments. Adding antibodies against Prss42/Tessp-2 and Prss43/Tessp-3 resulted in meiotic arrest at the stage when each protease was beginning to be translated. Furthermore, the number of apoptotic cells dramatically increased after the addition of these antibodies. These results strongly suggest that the three paralogous Prss/Tessp proteases play different roles in spermatogenesis and that Prss42/Tessp-2 and Prss43/Tessp-3 are required for germ cell survival during meiosis.

INTRODUCTION

Spermatogenesis is a precisely controlled process for generating spermatozoa. At the beginning of spermatogenesis, a certain percentage of spermatogonia, which undergo identical replications, enter meiosis to become primary spermatocytes [1]. These primary spermatocytes undergo meiosis I and divide into secondary spermatocytes and subsequently undergo meiosis II to become spermatids. Spermatids undergo spermiogenesis during which mature spermatozoa are formed by morphological and biochemical modifications [2, 3]. Spermatogenesis involves several important events including differentiation of primordial germ cells [4, 5], signaling between germ and Sertoli cells [6, 7], and substitution of histones to protamines after meiosis [8-10]. However, the molecular mechanisms involved in spermatogenesis are not

completely understood.

Proteases have been investigated in many tissues [11, 12], but limited studies have reported their functions related to male reproduction [13-15]. While some testicular proteases have been suggested to be involved in sperm maturation, capacitation, aggregation, and fertilization [16-18], others may have some roles in spermatids and spermatozoa [19-21]. These findings have revealed some of the protease functions after meiosis, but not during meiosis. However, it is evident that proteases have important functions in meiosis because mice that lack some protease inhibitors exhibit impaired meiosis [22, 23]. In addition, several proteases are expressed in spermatocytes [24-27]. Therefore, the functions of protease in germ cells during meiosis need to be elucidated.

For more insights into the functions of testicular proteases, we focused on testis-specific serine proteases that we recently cloned. We focused on serine proteases as they comprise the largest family of mammalian proteases [28]. We cloned four novel serine protease genes from eight-week-old mouse testes using reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers designed for the sequence conserved among serine proteases and by screening a mouse testis cDNA library. We originally named these four genes *testis-specific serine protease* (*Tessp*)*-1* through *Tessp-4* and deposited their primary sequences in the Genbank (accession numbers AB049453, AB052292, AB100999, and AB162857); subsequently, their gene symbols were officially assigned as *Prss41* through *Prss44*.

We previously reported that *Prss41/Tessp-1* was a glycosylphosphatidylinositol (GPI)-anchored membrane protein localized in the basal compartment of Sertoli cells, the plasma membrane of spermatogonia, and the Golgi apparatus of spermatocytes and spermatids [29, 30]. Wong et al. also studied the expression pattern of *Prss41/Tessp-1* as one of the genes constituting a 1.5-Mb serine protease gene cluster and obtained results consistent with those of our study [31]. In contrast, *Prss42/Tessp-2*, *Prss43/Tessp-3*, and *Prss44/Tessp-4* that form another gene cluster in chromosome 9F2-F3 (Fig. 1A) have not yet been characterized.

In this study, we investigated the expression patterns of these three proteases and attempted to determine their association with spermatogenesis by using an organ culture system. Our results demonstrated different expression patterns for these three proteases and suggested their importance in germ cell survival during spermatogenesis.

MATERIALS AND METHODS

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.

Phylogenetic tree construction

The amino acid sequences of mouse Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4 were compared with those of proteases from other species using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify their orthologs. Multiple alignments of amino acid sequences were performed using the ClustalW program. A neighbor-joining (NJ) tree was constructed and bootstrap analysis was performed using MEGA version 5.0 software [32].

RNA analyses

Northern blot, *in situ* hybridization, and quantitative RT-PCR (qRT-PCR) analyses were done as previously described [33]. Probes for Northern blot and *in situ* hybridization were obtained by RT-PCR using the primer pairs listed in Table 1. Primer sequences for qRT-PCR are also shown in Table 1.

Native testicular germ cell isolation and fractionation into different meiotic stages

The tunica albuginea was removed from each testis of an adult mouse. This tissue was placed in modified Eagle's medium (MEM) containing 0.5 mg/ml collagenase (Wako Pure Chemicals, Osaka, Japan) for approximately 30 min at 33°C in a water bath with occasional agitation. The tubules were separated from dispersed interstitial cells by unit gravity sedimentation for 5 min, and the supernatant was discarded. The tubules were treated again with MEM containing 0.5 mg/ml collagenase for 15 min at 33°C and washed thrice with PBS containing 1 mM EDTA. The tubules were cut into small pieces, and the germ cells inside them were dispersed by gentle pipetting. The germ cells were separated from the tubule pieces by unit gravity sedimentation, and the supernatant was collected as the germ cell fraction. Based on the marker

genes expression, this fraction included 84.3% of germ cells and major contaminants were Sertoli cells (Supplemental Table S1). For western blot analysis, the germ cells were fractionated into nuclear, membrane, and cytoplasmic fractions, according to the procedure described previously [34].

The germ cells were sorted into four meiotic stages, as described previously with slight modifications [35]. We used a JSAN cell sorter (Bay Bioscience, Kobe, Japan) equipped with a 375-nm ultraviolet laser. Cells were separated based on their staining intensities for Hoechst blue and red, which provided four groups of germ cells. A previous report showed that these four groups mainly contain spermatogonia, primary spermatocytes, secondary spermatocytes, or spermatids/spermatozoa [35]. We confirmed the purity of each fraction by microscopic observations and by examining the expression of marker genes for the meiotic stages by qRT-PCR. We chose *rnh2* (NLR family, pyrin domain containing 4C, BC120833), *sycp2* (synaptonemal complex protein 2, AK029735), *pten2* (transmembrane phosphatase with tensin homology, BC107329), and *prm1* (protamine 1, BC059733) as marker genes for spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, respectively [36, 37].

Preparation of specific Prss/Tessp antibodies

We amplified the nucleotide sequences corresponding to the amino acids for Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4 without highly hydrophobic regions by RT-PCR using the primers listed in Table 1. The recombinant Prss/Tessp proteins were produced and purified as previously described [34]. The purified protein was emulsified with Freund's complete adjuvant and injected intradermally into two-month-old female rabbits every two weeks. After three or four booster injections, the antisera were collected from the blood. All the antibodies used in this study were purified as described previously [34].

Preparation of whole cell extracts for western blot analysis

A whole testis, a whole liver, fractionated germ cells, and mature spermatozoa obtained from the cauda epididymis were homogenized or suspended in ice-cold PBS containing 1× proteinase inhibitor (Roche Molecular Biochemicals, Mannheim, Germany) and the same volume of 2× Lysis Buffer [0.1 M Tris-HCl (pH 7.5), 0.3 M NaCl, 0.5% sodium deoxycholate, 2% NP-40, 2 mM EDTA] that contained 1× proteinase inhibitor. The samples were mixed well and incubated on ice for 1 h. Then, the samples were sonicated to shear nucleic acids and centrifuged at 21,000×*g* for 10 min at 4°C, and the supernatant was collected as the

whole cell extract. Protein concentration was determined with a Pierce BCA protein assay reagent kit (Thermo Scientific, Rockford, IL, USA).

Western Blot Analysis

Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel under reducing conditions. Separated proteins were transferred to Immobilon transfer membranes (Millipore Corporation, Bedford, MA, USA) and blocked with Block Ace (Dainippon seiyaku, Osaka, Japan) for 1 h. Then, membranes were incubated with the purified Prss/Tessp antibodies at a final concentration of 10 μg/ml in PBS containing 0.1% Tween 20 (TPBS), for 1 h at room temperature. After washing four times with TPBS for 10 min each, the membranes were incubated with a secondary antibody, donkey anti-rabbit IgG HRP conjugate (1:5000 dilution, GE Healthcare Biosciences) with ExactaCruz^{TM} E Dilution Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 1 h. The membranes were washed again with TPBS four times, after which signals were detected using Immobilon Western reagent (Millipore Corporation). To confirm signal specificity, we conducted the same experiment using antibodies that had been pre-incubated with each of the Prss/Tessp antigens.

Immunohistochemistry

Testes harvested from six- to eight-week-old mice were fixed in Bouin's solution for 18 h at 4°C, embedded in paraffin, and cut into 5-μm sections. Sections were mounted on MAS-coated slides (Matsunami, Osaka, Japan), deparaffinized with xylene and rehydrated in a series of ethanol solutions, and boiled in 10 mM citric acid (pH 6.0) for 5 min. The slides were then treated with 3% hydrogen peroxide (Wako Pure Chemicals) in PBS for 5 min and washed thrice with PBS for 5 min each. Non-specific binding sites were blocked with Block Ace (Dainippon seiyaku) for 1 h at room temperature, after which the sections were incubated with the Prss/Tessp antibodies at a final concentration of 100 μg/ml for 16 h at room temperature. The slides were washed with TPBS thrice and incubated with donkey anti-rabbit IgG HRP conjutgate (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.

Effect of pohsphatidylinositol-specific phospholipase C (PI-PLC) treatment

A membrane fraction of germ cells from eight-week old mouse testes was prepared as above and resolved in 50 mM Tris-HCl (pH 7.4). The sample was treated with or without PI-PLC (Sigma, St. Louis, MO, 0.2U/ml) for 1 h at 37°C with mixing every fifteen minutes. The samples were then spun at 100,000×*g* for 15 min and the supernatant was collected for western blot analysis. Urokinase-type plasminogen activator receptor (uPAR) was detected as a positive control with the antibody purchased from Sino Biological Inc. (Beijing, China).

Organ culture of testis fragments with the Prss/Tessp antibodies

Organ culture for a mouse testis was performed using established methods with modifications [38, 39]. Testes were harvested from two-week-old mice and cut into small fragments (approximately 2 mm × 2 mm). The tissue pieces were placed on a Nuclepore Track-Etch Membrane (0.2 μm of pore-size, Whatman, GE Healthcare Biosciences) and cultured in DMEM containing 10% FBS with the purified Prss/Tessp antibodies or the antibodies pre-incubated with respective antigens. The antibodies were used at a final concentration of 50 μg/ml. Tissues were cultured for two weeks with medium changes every three days. Then, the tissue pieces were fixed with Bouin's solution for 18 h at 4°C, and paraffin sections were prepared, and stained with hematoxylin. Alternatively, total RNA was isolated from the testis pieces after organ culture and used for qRT-PCR to investigate the expression of marker genes for the different meiotic stages.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The paraffin sections prepared after organ culture were also used for a TUNEL assay. Deparaffinized slides were treated with 20 μg/ml proteinase K in 10 mM Tris-HCl (pH 7.4) for 15 min and washed with PBS twice. Staining of apoptotic cells was performed using *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. A TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP was incubated on the slides in a moisture chamber for 1 h at 37°C. After three washes with PBS, the slides were counterstained with Hoechst 33342 and observed under a fluorescence microscope.

Statistical analysis

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

RESULTS

The three Prss/Tessp genes are presumptive paralogs

We first analyzed the evolutionary relationships between the three *Prss/Tessp* genes (Fig. 1A). We compared the amino acid sequences of these genes with the NCBI database using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We found orthologs for the *Prss42/Tessp-2*, *Prss43/Tessp-3*, and *Prss44/Tessp-4* genes among fifteen, seven, and thirteen eutherian species, respectively. We could not find any gene that was homologous to these three genes among marsupials and monotremes. We then examined the synteny of this locus by comparing the gene order among those species for which genome sequences have been determined (Fig. 1B). Although the number of *Prss* genes in this cluster differed among these species, gene synteny was well conserved, except for pig. These results suggested that the Prss genes in this cluster had evolved by gene duplications after eutheria diverged from marsupials.

Next, we compared the three *Prss/Tessp* genes with other murine serine proteases. We constructed a phylogenetic tree using the serine proteases present in two gene clusters, one at chromosome 17A3.3 including *Prss41/Tessp1* [31] and the other at chromosome 9F2-F3 including the three Prss/Tessp genes. As shown in Fig. 1C, three groups were formed; group II and group III included all the genes from the cluster at chromosome 17A3.3. Group I included the three Prss/Tessp genes. The bootstrap value for the branch point between Prss43/Tessp3 and the other two Prss/Tessps was 97 (Fig. 1C), which suggested that these three genes arose through ancient gene duplication. Taken together, the three *Prss/Tessp* genes are presumptive paralogs.

mRNAs of the three Prss/Tessp genes are specifically expressed at different levels in the testis

To examine the mRNA distribution of the three Prss/Tessp genes, we used northern blot analysis with total RNAs extracted from fifteen mouse tissues (Fig. 2A). We detected specific signals for all the *Prss/Tessp*

genes only in the testis. Specific bands were observed for *Prss42/Tessp-2* and *Prss43/Tessp-3* at the positions 1.65 kb and 2.2 kb, respectively, whereas a broad band was detected for *Prss44/Tessp-4* at the position between 1.5 kb and 2.3 kb. This was consistent with the *Prss44/Tessp-4* gene having two transcripts whose molecular sizes were predicted to be 1.7 kb and 2.1 kb, as indicated in the Ensembl database (http://www.ensembl.org/).

Next, we examined mRNA expression at different developmental stages of the mouse testis (7, 14, 21, 28, and 56 days after birth) by qRT-PCR (Fig. 2B). We detected all the *Prss/Tessp* mRNAs at all stages, except for 7 days after birth. At day 14, a trace level of each *Prss/Tessp* mRNA was detected and *Prss42/Tessp-2* was expressed at a lower level than the others (Fig. 2B, inset). All the mRNA levels increased dramatically at day 21, reached a peak at day 28, after which the expressions of *Prss43/Tessp-3* and *Prss44/Tessp-4* decreased. The expression of *Prss42/Tessp-2* mRNA remained high at day 56. These results suggested that the three *Prss/Tessp* genes were mainly transcribed at the late pachytene spermatocyte stage and later because the 14-day-old testis does not yet contain these cell types [40].

The qRT-PCR results also indicated that the three protease genes were expressed at different levels. Since the amplification efficiency of the three *Prss/Tessp* primer pairs was equivalent and the primers specifically amplified their corresponding target genes, we were able to compare their expression levels by this analysis. At 21, 28, and 56 days after birth, *Prss42/Tessp-2* mRNA was always expressed at the highest level, followed by *Prss43/Tessp-3* and *Prss44/Tessp-4* (Fig. 2B). In the 56-day-old testis, *Prss42/Tessp-2* and *Prss43/Tessp-3* had 6.3-fold and 1.6-fold higher mRNA expression levels compared with that of *Prss44/Tessp-4*.

mRNAs of the three Prss/Tessp genes are localized in primary and secondary spermatocytes

We performed *in situ* hybridization with the testes from 28-day-old mice to determine the localization of the *Prss/Tessp* mRNAs (Fig. 3). The 28-day-old mouse was chosen because all *Prss/Tessp* mRNAs showed their highest expression levels at this age (Fig. 2B). We detected signals for all three *Prss/Tessp* mRNAs in primary spermatocytes with antisense probes (Fig. 3A, C, E, G, I, K) whereas no signal was observed in the sections hybridized with sense probes (Fig. 3B, F, J). We also investigated their localizations using testes from 14- and 56-day-old mice. Very weak signals were detected for *Prss43/Tessp-3* and *Prss44/Tessp-4* in spermatocytes of the 14-day-old testis, but no signal was observed for *Prss42/Tessp-2* (data not shown). This was consistent with our qRT-PCR data showing that *Prss42/Tessp-2* was detected at a lower level in the 14-

day-old testis (Fig. 2B, inset). In comparison, we detected all *Prss/Tessp* mRNA signals in primary spermatocytes of the 56-day-old mouse testis, although the signal intensities appeared to be weaker than those of 28-day-old mouse testis, particularly for *Prss44/Tessp-4* (Fig. 3D, H, L). At day 56, the seminiferous tubules without condensing spermatids seemed to contain the signals for all Prss/Tessps. This suggested that these mRNAs were present in spermatocytes at the seminiferous epithelial stages IX-XII when late pachytene spermatocytes and secondary spermatocytes appeared.

To further assess the cell types that expressed the *Prss/Tessp* mRNAs, we used qRT-PCR with RNA purified from sorted germ cells (Fig. 4A). We isolated germ cells from native testicular cells and fractionated them with a cell sorter method described previously [35]. We collected four cell fractions and validated that each fraction corresponded to the expected cell type by examining the expression of a marker gene for each meiotic stage. These markers were *rnh2*, *sycp2*, *pten2*, and *prm1* for spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, respectively [36, 37]. This showed that *rnh2*, *sycp2*, *pten2*, and *prm1* were predominantly expressed in fractions #1, #2, #3, and #4, respectively (Fig. 4B), which indicated that we had successfully fractionated the germ cells into each of the meiotic stages. We also confirmed the cell type of each fraction by fluorescence microscopy (Supplemental Fig. S1).

Then, we investigated the *Prss/Tessp* mRNA expressions in these fractions. By qRT-PCR analysis, all three *Prss/Tessp* mRNAs were expressed not only in primary spermatocytes (fraction #2), but also in secondary spermatocytes (fraction #3; Fig. 4C). For *Prss42/Tessp-2* and *Prss43/Tessp-3*, weak signals were also detected in fraction #4 that primarily included spermatids and spermatozoa, although this could have been due to contamination by spermatocytes. Taken together with the results of *in situ* hybridization and qRT-PCR with various developmental stages of testes, all the *Prss/Tessp* mRNAs were mainly expressed at the stages from the late pachytene primary spermatocyte to the secondary spermatocyte.

Generation of specific antibodies against the three Prss/Tessp proteins

For further analysis, we generated and purified polyclonal antibodies against the three Prss/Tessp proteins. We first checked the quality of the purified antibodies by western blot analysis using a whole cell extract of mouse testis. We observed single bands for Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4 at the positions 37 kDa, 42 kDa, and 40 kDa, respectively (Fig. 5A, lane 1). To confirm the specificity of these signals, we did the same experiment using antibodies that had been pre-incubated with recombinant

Prss/Tessp antigens. As a result, the bands disappeared in all these experiments (Fig. 5A, lane 2), which indicated that each antibody specifically recognized a Prss/Tessp protein. We also confirmed that each antibody did not recognize other Prss/Tessp proteins by western blot using the antibody preabsorbed with the other Prss/Tessp antigens (Supplemental Fig. S2), and these antibodies did not recognize any proteins in the liver (Fig. 5B).

Many proteases were produced as precursors or zymogens and activated by the release of their propeptides. The detected molecular masses of the three Prss/Tessp proteins corresponded to those expected from the amino acid sequences with their propeptides. This suggested that these antibodies primarily recognized the precursor forms of the proteases and/or the whole testis extract contained far more proforms than the activated forms.

Protein localization is different between the three Prss/Tessp proteases

We used immunohistochemistry to determine the localization of the three Prss/Tessp proteins in the mouse testis. Unfortunately, the antibody against Prss44/Tessp-4 did not work well for this analysis. We could only detect specific signals for the Prss42/Tessp-2 and Prss43/Tessp-3 proteins (Fig. 6A, C). Interestingly, despite their similar mRNA distributions (Figs. 2-4), the protein localizations of Prss42/Tessp-2 and Prss43/Tessp-3 were different. For Prss42/Tessp-2, the signal was mainly detected in round spermatids at all the seminiferous epithelial stages, but not in spermatocytes (Fig. 6E-G). In contrast, Prss43/Tessp-3 was expressed in germ cells at the stages from late pachytene spermatocytes to spermatids (Fig. 6H-J). No signal was observed for both proteases in Leydig cells, and the antibodies pre-incubated with the antigens did not produce any signal (Fig. 6B, D). As for Sertoli cells, we purified the cell from 10-day-old testes and used RT-PCR and western blot. The results clearly demonstrated that the three Prss/Tessp genes were neither transcribed nor translated in Sertoli cells (Supplemental Fig. S3).

To further explore the protein localization of the Prss/Tessp proteases, we used western blot analysis for testicular germ cells fractionated into different meiotic stages (Fig. 6K). Because the three *Prss/Tessp* mRNAs were expressed in primary and secondary spermatocytes (Figs. 2-4), we excluded spermatogonia from this analysis. Instead, we used mature spermatozoa collected from the cauda epididymis. This showed that Prss42/Tessp-2 were in the fractions that included secondary spermatocytes and spermatids/spermatozoa. The Prss43/Tessp-3 protein was produced at the stages from primary spermatocytes through

spermatids/spermatozoa. For Prss44/Tessp-4, it was detected at the stages from primary spermatocytes through spermatids/spermatozoa. None of the Prss/Tessp proteins was detected in mature spermatozoa. Although we could not completely rule out the possibility that the proteases were present in a spermatozoon because the epitope could be hidden in this special cell, the results suggested that these proteases' functions were inside the testis.

Notably, in addition to the signals corresponding to their proforms, additional bands were detected for Prss42/Tessp-2 and Prss43/Tessp-3 at the positions 31 kDa and 29 kDa, respectively (Fig. 6K). In case of serine proteases, the typical cleavage site for activation was the amino side of the Ile-Val-Gly-Gly or corresponding sequence [41], and we could determine the sites for Prss42/Tessp-2 and Prss43/Tessp-3, as shown in Supplemental Fig. S4. The calculated molecular masses of their activated forms were similar to those of the additional bands observed in our experiment (Fig. 6K), which suggested that we might detect the active forms of Prss42/Tessp-2 and Prss43/Tessp-3.

Both Prss42/Tessp-2 and Prss43/Tessp-3 are GPI-anchored membrane proteins

The results of immunohistochemistry also indicated that the Prss42/Tessp-2 and Prss43/Tessp-3 proteins were not localized at the nucleus, although it was difficult to determine whether they were localized at the membrane or in the cytoplasm (Fig. 6A, C). To determine the subcellular localization of the Prss/Tessp proteins, we fractionated germ cells into nuclear, membrane, and cytoplasmic subfractions and then used western blot analysis. Signals of marker proteins for these fractions (Histone H3, Cdh2, and Tuba1a) indicated that our fractionation was successful (Fig. 7A). We detected specific signals in the membrane and cytoplasmic fractions for Prss42/Tessp-2, only in the membrane fraction for Prss43/Tessp-3, and in the cytoplasmic fraction for Prss44/Tessp-4 (Fig. 7A). We also detected putative activated forms of Prss42/Tessp-2 and Prss43/Tessp-3 from the membrane fraction.

Because both Prss42/Tessp-2 and Prss43/Tessp-3 were associated with the membrane, we assessed what kinds of membrane proteins they were. Based on their amino acid sequences that had hydrophobic regions at their C-termini (Supplemental Fig. S4), these proteases were expected to be GPI-anchored proteins. To test this possibility, we treated the membrane fraction of testicular germ cells with PI-PLC. If these proteases were GPI-anchored proteins, the PI-PLC treatment would release them from the membrane. Using the supernatant after PI-PLC treatment, we detected specific signals for both Prss/Tessps, whereas no signals

were observed without the enzyme (Fig. 7B). uPAR was used as a positive control to show that we could successfully digest a GPI-anchored protein. This indicated that both Prss42/Tessp-2 and Prss43/Tessp-3 were GPI-anchored membrane proteins.

Antibodies against Prss42/Tessp-2 and Prss43/Tessp-3 induce meiotic arrest during in vitro *organ culture of testis fragments*

To examine the roles of the Prss/Tessp proteases in spermatogenesis, we used organ culture of mouse testis fragments [38, 39]. A testis was harvested from a two-week-old mouse, cut into small fragments, and these tissue pieces were cultured either with the purified Prss/Tessp antibodies (Fig. 8B, D) or with antibodies preabsorbed with the Prss/Tessp antigens (Fig. 8A, C). We did not use the Prss44/Tessp-4 antibody in this experiment because it did not work for immunohistochemistry and the Prss44/Tessp-4 protein was located in the cytoplasm where this antibody would not reach (Fig. 7A).

After culture for two weeks, we observed apparent decreases in germ cells in many seminiferous tubules after adding the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies. While two or three layers of germ cells were observed in the seminiferous tubules of the control tissues (Fig. 8A, C), a few germ cells were present when the tissues were cultured with these antibodies (Fig. 8B, D). Indeed, adding the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies decreased the cell numbers per seminiferous tubule to 36% and 48% of control sections, respectively (Fig. 8E). These results suggested that adding these Prss/Tessp antibodies had impaired meiosis.

Because it was difficult to determine what types of germ cells were included in the seminiferous tubule by microscopic observations of the tissue sections, we used qRT-PCR with cDNA prepared from the cultured tissue (Fig. 8F, G). We checked the expression level of a marker gene for each meiotic stage to determine the cell type included in the tissue after culture. If meiosis had proceeded normally, we would have detected the expression of all the marker genes. Indeed, we could detect the expression of the four marker genes in the tissues cultured with preabsorbed antibodies, which indicated that the germ cells had successfully undergone meiosis in the control experiments.

When we cultured the testis fragments with the Prss42/Tessp-2 antibody, we detected the expression of all the marker genes at similar levels to the control, except for *prm1* (Fig. 8F), which indicated that the tissues included spermatogonia and primary and secondary spermatocytes, but not spermatids. In addition, *pten2*

expression increased significantly, which indicated that the population of secondary spermatocytes increased after adding the Prss42/Tessp-2 antibody. This strongly suggested that this antibody held meiosis at the secondary spermatocyte stage.

Culture with the Prss43/Tessp-3 antibody resulted in a dramatic decrease in *pten2* and *prm1* and an increase in *sycp2* expression (Fig. 8G). This indicated that the tissue cultured with the Prss43/Tessp-3 antibody contained more primary spermatocytes and no germ cells at later stages. Therefore, we concluded that adding the Prss43/Tessp-3 antibody resulted in meiotic arrest at the primary spermatocyte stage.

We also investigated the expression of marker genes for Leydig and Sertoli cells as well as for germ cells and confirmed that only germ cells were affected by adding the antibodies (Supplemental Fig. S5A, B). In addition, the antibody against medaka MT2-MMP [42], which never recognized any proteins in the mouse testis, had no effect on the marker genes expression (Supplemental Fig. S5C). Collectively, each antibody induced meiotic arrest at that stage when the corresponding protein began to be translated, i.e, the secondary spermatocyte for Prss42/Tessp-2 and the primary spermatocyte for Prss43/Tessp-3 (Fig. 6K).

Adding the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies causes germ cells apoptosis

Because adding the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies resulted in a decrease in cell number per seminiferous tubule (Fig. 8E), we tested for the possibility that these antibodies induced germ cells apoptosis. We performed TUNEL assay using the sections of the cultured tissues. This showed that the numbers of TUNEL-positive cells increased in the tissues cultured with the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies compared with those in the control sections (Fig. 9A, B). The signals appeared to be located inside the seminiferous tubules, which suggested that most of the apoptotic cells were germ cells. Therefore, organ culture of testis fragments with the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies probably induced meiotic arrest and germ cells apoptosis.

DISCUSSION

Various proteases are expressed in germ cells during spermatogenesis, although most of them function in spermatids and spermatozoa and are involved in fertilization [13-21, 43-46]. We analyze the three Prss/Tessp

proteases that are expressed during meiosis. These proteases were transcribed at similar meiotic stages, but translated differently and located in different subcellular fractions. *In vitro* organ culture revealed that Prss42/Tessp-2 and Prss43/Tessp-3 were required for the progression of meiosis at different meiotic stages. These data provide new insights into the functions of protease in the testis, especially during meiosis.

The three Prss/Tessp proteases have distinct roles in spermatogenesis

Although all three *Prss/Tessp* mRNAs were expressed in primary and secondary spermatocytes (Figs. 2-4), the localization of their translated products were different (Fig. 6). In particular, Prss42/Tessp-2 protein was detected in secondary spermatocytes and spermatids, which indicated that *Prss42/Tessp-2* mRNA was under translational arrest in primary spermatocytes as is often observed for the genes expressed during spermatogenesis [47-50]. This also suggested that Prss42/Tessp-2 functioned at the late stages of meiosis and/or during spermiogenesis. In contrast, the cell types that produced the Prss43/Tessp-3 and Prss44/Tessp-4 proteins generally coincided with those that expressed their mRNAs (i.e., primary and secondary spermatocytes) and their production was retained in spermatids. Thus, Prss43/Tessp-3 and Prss44/Tessp-4 probably function at earlier meiotic stages than Prss42/Tessp-2. This suggests that Prss42/Tessp-2 has a distinct role in meiosis compared with those of Prss43/Tessp-3 and Prss44/Tessp-4.

It is worth noting that the three Prss/Tessp proteases exhibited different subcellular distributions (Fig. 7A). Prss42/Tessp-2 was located at the membrane and in cytoplasm, whereas Prss43/Tessp-3 and Prss44/Tessp-4 were exclusively found in the membrane and cytoplasmic fractions, respectively. Based on their amino acid sequences, these proteases were predicted to be GPI-anchored membrane proteins (Supplemental Fig. S4), and indeed, Prss42/Tessp-2 and Prss43/Tessp-3 were associated with the membrane by GPI (Fig. 7B). Thus, it was surprising that Prss42/Tessp-2 and Prss44/Tessp-4 were found in the cytoplasm. It is possible that we detected these proteins inside intracellular compartments, such as the Golgi apparatus and the endoplasmic reticulum, although it is also possible that these proteases actually do exist in the cytoplasm.

Several putative GPI-anchored proteins have been reported in the testis, but most of them are localized at the sperm membrane and work during fertilization [51-53]. After meiosis, a famous GPI-anchored protein, uPAR, is found at Sertoli-germ cell contacts and assumed to be involved in the regulation of spermatid adhesion [12]. During meiosis, another testis-specific GPI-anchored protease, Prss41/Tessp-1, is localized in the Golgi-apparatus of spermatocytes and spermatids [29, 30]. Notably, one putative GPI-anchored protein,

Ly6k, is present in the testis as two different forms and one is localized in the cytoplasm of germ cells [54]. At present, we have no explanation as to why Prss42/Tessp-2 and Prss44/Tessp-4 were detected in the cytoplasmic fraction. Nevertheless, their distinctive subcellular localizations suggest that these three Prss/Tessp proteases have functions that are different from one another.

It was also interesting that we primarily detected the proforms of each protease by western blot analysis. The molecular masses observed with a whole testis extract corresponded to those calculated from the amino acid sequences with a propeptide for each Prss/Tessp (Fig. 5). For Prss42/Tessp-2 and Prss43/Tessp-3, we also detected signals for lower molecular masses of 31 kDa and 29 kDa, respectively, when we used the membrane fraction of germ cells and the germ cell extracts for different meiotic stages (Figs. 6K, 7A). These molecular masses were in good agreement with those calculated from their putative activated forms, 28.4 kDa for Prss42/Tessp-2 and 29.4 kDa for Prss43/Tessp-3 (Supplemental Fig. S4).

However, a hydrophobic C-terminal region of a GPI-anchored protein is cleaved prior to protein maturation. Based on the PRED GPI program (http://gpcr.biocomp.unibo.it /predgpi/, [55]), Prss42/Tessp-2 and Prss43/Tessp-3 would be cleaved at Tyr^{306}-Ser^{307} and Ser^{355}-Gln^{356} (Supplemental Fig. S4), and the resulting molecular masses would be 25.3 kDa and 26.5 kDa, respectively. We observed signals for higher molecular masses (Figs. 6K, 7A), which suggested that the mature proteases may be N-glycosylated. In fact, some glycosylation sites were predicted from their sequences (Supplemental Fig. S4). In any case, we think that the signals for lower molecular masses could be the activated forms of Prss42/Tessp-2 and Prss43/Tessp-3.

As indicated above, the expression patterns of the three Prss/Tessp proteins were different in terms of the meiotic stages when they appeared, their subcellular distributions, and the presence of activated forms, all of which strongly suggests that they play different roles in spermatogenesis. To determine the function of a protease, it is important to characterize its enzymatic activity and identify its physiological substrates. We attempted to obtain Prss/Tessp proteases that maintained their enzymatic activities by overexpressing them in some bacterial and mammalian cells and by attempting to purify native Prss/Tessp proteins from testicular germ cells. However, to date, none of these attempts has been successful (data not shown), perhaps because the Prss/Tessp proteases have very strict substrate specificities or have no enzymatic activities. Additional analyses will be necessary to determine the biochemical characteristics of these Prss/Tessp proteases.

The Prss42/Tessp-2 and Prss43/Tessp-3 are involved in germ cell survival during meiosis

To determine the functions of the Prss/Tessp proteases, we conducted organ culture using testis fragments. Several methods for *in vitro* spermatogenesis have been published. For most of these, meiosis successfully proceeds to the spermatid stage [56-59]. Because the Prss/Tessp proteins were present in spermatocytes and spermatids, organ culture was a suitable system to investigate their functions.

We used antibodies against Prss42/Tessp-2 and Prss43/Tessp-3 and expected that these would block these protease functions by specifically binding to their proteins. Indeed, they were effective for the cells inside seminiferous tubules. Adding these antibodies dramatically reduced the number of cells inside the seminiferous tubules (Fig. 8A-E) and most of the apoptotic cells we observed were inside these tubules (Fig. 9). Moreover, the expression levels of marker genes for Leydig and Sertoli cells were similar between the tissues cultured with and without these antibodies (Supplemental Fig. S5A, B). These results indicated that adding the Prss/Tessp antibodies induced the apoptosis of germ cells, but not of somatic cells.

The Prss/Tessp antibodies also induced meiotic arrest. When using the Prss42/Tessp-2 antibody, meiosis stopped at the secondary spermatocyte, and the Prss43/Tessp-3 antibody held meiosis at the primary spermatocyte stage (Fig. 8F, G). These indicated that the antibodies induced meiotic arrest at the stages when the corresponding protease began to be produced. There are some studies reporting that meiotic arrest could lead to germ cell apoptosis [23, 60, 61]. Therefore, we conclude that the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies induced meiotic arrest in primary and secondary spermatocytes, respectively, which could result in their apoptosis.

In the organ culture experiments, we observed the five- and four-fold increases of *pten2* and *sycp2* expression by adding the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies, respectively (Fig. 8F, G). This is possibly because germ cells at earlier stages would continue with meiosis, and thus, secondary or primary spermatocytes would accumulate. This could increase the population of the arrested cells and could result in the several-fold increase of the marker genes expression.

How the Prss/Tessp antibodies induced meiotic arrest is unclear. However, because the antibodies could not get inside a cell, the membrane-bound forms of Prss42/Tessp-2 and Prss43/Tessp-3 outside a cell will be affected. One possibility is that these proteases are involved in the germ cell migration from the basal lamina to the inner lumen of seminiferous tubules [62]. They may degrade the extracellular matrix (ECM) to promote migration, as is the case for MT1-MMP that degrades the ECM between Sertoli cells and elongated

463 spermatids to emit mature spermatozoa [63]. Alternatively, the Prss/Tessp proteases may degrade several
464 types of cell-cell junctions, such as tight, anchoring, or gap junctions between germ and Sertoli cells [64, 65].
465 In this scenario, the Prss/Tessp antibodies may interfere with the degradation of the ECM or junction proteins,
466 after which germ cells may be unable to migrate into the inner lumen. This could induce meiotic arrest and
467 subsequently, promote germ cell apoptosis.
468 However, it is possible that the Prss/Tessp proteases may be related to other functions. For example, they
469 may activate some signaling proteins. Bone morphogenic proteins (BMPs) could be the candidates because
470 some BMPs are present in the testis and play crucial roles in spermatogenesis [66, 67]. Prss/Tessps may also
471 be involved in the activation of other membrane proteases as was suggested for trypsin in the eel testis [68].
472 Alternatively, they may have some roles irrespective their protease activities such as growth activities of PA
473 in tumors [69]. More studies are needed to examine whether the junction proteins or the testicular ECM are
474 targets of the Prss/Tessp proteases or whether these proteases have other functions. Nevertheless, our current
475 results strongly suggest that the Prss/Tessp proteases are critical for meiosis.
476
477 In summary, the paralogous Prss/Tessp proteases are expressed differently in testicular germ cells during
478 meiosis, which suggests that they have distinct functions during meiosis. In the organ culture experiments
479 using testis fragments, the antibodies against Prss42/Tessp-2 and Prss43/Tessp-3 induced meiotic arrest and
480 germ cell apoptosis at the stages when these proteases began to be translated. Our results highlight the
481 importance of proteases during meiosis in the mammalian testis.

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FIGURE LEGENDS

FIG. 1. Genomic structure of the Prss/Tessp gene cluster and their phylogenetic relationships. **A**) (top) A schematic drawing of the genomic structure of the Prss/Tessp and neighboring genes at the mouse chromosome 9F2-F3. An arrow on each gene shows the direction of transcription. (bottom) Exon-intron structures of the three Prss/Tessp genes. Boxes indicate exons of the Prss/Tessp genes, and filled and unfilled boxes in the exons represent coding and noncoding regions, respectively. **B**) Synteny of the Prss/Tessp genes among thirteen eutherian species. An ortholog of each Prss/Tessp gene is shown with the same color as the corresponding gene. Gene names are written based on the Ensembl database. The gene order at the Prss/Tessp locus is indicated for the species that have the orthologs and whose genome sequences have been already determined. The phylogenetic relationships of these species were illustrated based on a report by Murphy et al. [70]. **C**) A phylogenetic tree of the genes present in two serine protease gene clusters, one at

chromosome 17A3.3 and the other at chromosome 9F2-F3 in mouse. Sequences of the mouse Prss genes were obtained from the Ensembl genome browser (http://www.ensembl.org/) and a neighbor-joining tree was constructed using their amino acid sequences. Three groups were formed as marked with I, II, and III. The number written in the tree represents the bootstrap value of each branch, and only bootstrap values larger than 50% are shown.

FIG. 2. Distribution of the Prss/Tessp transcripts. **A**) Northern blot analysis of the three *Prss/Tessp* mRNAs in various mouse tissues. Specific bands were detected for all three *Prss/Tessp* genes only in the testis. *Actb* and *Gapdh* were used as controls. The experiment was repeated three times, and the representative result is shown. **B**) qRT-PCR analysis of the *Prss/Tessp* genes during postnatal testicular development. Total RNAs isolated from testes of 7-, 14-, 21-, 28-, and 56-day-old mice were used for cDNA synthesis, and qRT-PCR was performed using the ABI Prism 7300 real-time PCR system. In the inset, the expression level of the 14-day-old testis is shown in larger scale. The Prss/Tessp expression levels were normalized to *Gapdh*. Values are means ± S.D.

FIG. 3. Localization of the Prss/Tessp transcripts in the mouse testis. **A**-**L**) *In situ* hybridization analysis of the *Prss/Tessp* genes in the mouse testis. Localization of the *Prss/Tessp* mRNAs was determined with the mouse testes harvested from 28- (A-C, E-G, I-K) and 56-day-old mice (D, H, L). For the 28-day-old testis, sections hybridized with antisense (A, E, I) and sense probes (B, F, J) are shown, and the signals with antisense probes are also indicated with higher magnification (C, G, K). For the 56-day-old testis, a magnified picture of each section hybridized with an antisense probe is shown (F, J, N). Sections were counter-stained by methylgreen (C, D, G, H, K, L). Positive signals (*purple*) for all three *Prss/Tessp* mRNAs were detected in late pachytene primary spermatocytes. The experiment was repeated three times, and the representative pictures are shown. Scale bars = 100 μm.

FIG. 4. The Prss/Tessp mRNA expressions in testicular germ cells sorted into four meiotic stages. **A**) A flow cytometric analysis. When the cells were plotted by their staining intensities for Hoechst blue (y axis) and red (x axis), four groups were formed as shown by ovals. Each group of cells was collected and designated as

fraction #1 through #4 as indicated. **B**) The expression of marker genes for meiotic stages in the sorted germ cell fractions. The *rnh2*, *sycp2*, *pten2*, and *prm1* genes were used as markers of spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, respectively. The data was normalized to *Gapdh*. The fractions #1, #2, #3, and #4 were found to primarily contain spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, respectively. Values are means ± S.D. **C**) The expression of *Prss/Tessp* mRNAs in the sorted germ cell fractions. qRT-PCR was performed as in B. Values are means ± S.D.

FIG. 5. Evaluation of antibodies against the Prss/Tessp proteins. **A**) Western blot analysis with a whole testis extract. A whole cell extract was prepared from an eight-week-old mouse testis and the proteins were transferred to a membrane and reacted with the Prss/Tessp antibodies (lane 1) or the antibodies that had been preincubated with their antigens (lane 2). A specific signal was detected with each antibody at the indicated position, but not with the preabsorbed one. **B**) Western blot analysis with testis and liver extracts. A whole cell extract was prepared from an eight-week-old mouse testis and liver and western blot analysis was conducted as in A. Specific signals were only detected from the testis protein extract.

FIG. 6. Localization of the Prss/Tessp proteins. **A-J**) Immunohistochemical analysis of Prss42/Tessp-2 and Prss43/Tessp-3. Positive signals (*red*) were detected primarily in spermatids for Prss42/Tessp-2 (A, E-G) and in spermatocytes and spermatids for Prss43/Tessp-3 (C, H-J). The sections were counter-stained with hematoxylin (E-J), and the seminiferous epithelial stages were indicated in Roman numbers. No signal was observed with the preabsorbed antibodies (B, D). Secondary spermatocytes are indicated in *arrows* (G, J). Scale bars = 100 μm. **K**) Western blot analysis with a whole cell extract of testicular germ cells at each meiotic stage. Testicular germ cells were sorted into four meiotic stages as in Fig. 4. Mature spermatozoa were harvested from the cauda epididymis. Specific signals were detected at the indicated positions. Actb was used as a control.

FIG. 7. Subcellular localization of Prss/Tessp proteins. **A**) Western blot analysis with the nuclear, membrane, and cytoplasmic fractions of the mouse testicular germ cells. The positions of molecular size markers are

indicated at right. Histone H3, Cdh2, and Tuba1a were used as markers for the nuclear, membrane, and cytoplasmic fractions, respectively. **B**) Western blot analysis of membrane proteins after PI-PLC treatment. The membrane obtained from native testicular germ cells was treated with (+) or without PI-PLC (-) and used for western blot analysis with the Prss42/Tessp-2 and Prss43/Tessp-3 antibodies. uPAR is known to be a GPI-anchored protein in the testis and used as a positive control.

FIG. 8. Organ culture of testis fragments with the Prss/Tessp antibodies. **A**-**D**) Small testis pieces were prepared from the 2-week-old mice and cultured with the Prss42/Tessp-2 or Prss43/Tessp-3 antibody. As a control, the tissue pieces were also cultured with the antibodies that were preincubated with their antigens. Pictures of the tissue sections cultured with the Prss/Tessp antibody (B, D) and the preabsorbed antibody (A, C) are indicated. The experiment was repeated six times for Prss42/Tessp-2 and seven times for Prss43/Tessp-3, and representative pictures are shown. Scale bars = 50 μm. **E**) The number of germ cells per seminiferous tubules. Two or three seminiferous tubules from each of six or seven individual experiments were selected randomly and the number of germ cells was counted. Values are means ± S.D. (**, $p < 0.01$ compared with control, $n = 12, 15$). **F** and **G**) The expression of marker genes in the testis fragments cultured with Prss42/Tessp-2 antibody (F) or Prss43/Tessp-3 antibody (G). The expression of marker genes for different meiotic stages was examined by qRT-PCR. Relative expression is shown as fold difference calculated relative to the expression in the control tissues that were cultured with the preabsorbed antibody. control, fragments cultured with the preabsorbed Prss/Tessp antibodies; antibody, fragments cultured with the Prss/Tessp antibodies. The data are presented as mean ± S.E.M. (**, $p < 0.01$, $n = 6$). *ND*; not detected.

FIG. 9. Increased apoptosis in the testis fragments cultured with the Prss/Tessp antibodies. **A)** TUNEL assays of the testis fragments cultured with the Prss42/Tessp-2 antibody. Testis pieces from 2-week-old mice were cultured with the Prss42/Tessp-2 antibody (*bottom*) or with the antibody that had been preincubated with its antigen as a control for 2 weeks (*top*). Paraffin sections were prepared from the cultured tissues, and the cell undergoing apoptosis were labeled using TdT and fluorescein-dUTP (*green*). Cell nuclei were counterstained with Hoechst 33342 (*blue*). Scale bars = 100 μm. **B)** TUNEL assay of the testis fragments cultured with the Prss43/Tessp-3 antibody. The testis fragments were cultured with the Prss43/Tessp-3 antibody and apoptotic

cells were detected as in A. *Scale bars* = 100 μm.

Table 1. Primers used in this study

Designation	forward	reverse
Northern blot analysis and *in situ* hybridization		
Prss42/Tessp-2	5’-ATG CAT GTC TGT GGA GGT TC-3’	5’-CTG AAA GTG TGA CCC TGG TC-3’
Prss43/Tessp-3	5’-AGA TTC CAG GAT CAG GGT CT-3’	5’-GAC AGG GTC ACA CTT TCA G-3’
Prss44/Tessp-4	5’-CCA TGG TCC TCT TGT TTC TG-3’	5’-CAC AAT CCT TCA CTC CAG GT-3’
Gapdh	5’-CAT GGC CTT CCG TGT TCC TA-3’	5’-CCT GCT TCA CCA CCT TCT TGA-3’
Actb	5’-ACA TCC GTA AAG ACC TCT ATG-3’	5’-TAA AAC GCA GCT CAG TAA CAG T-3’
qRT-PCR		
Prss42/Tessp-2	5’-GTC AAG AGA GGC ATG GTC TG-3’	5’-AAC TCC TAC CTG CAC CCA TT-3’
Prss43/Tessp-3	5’-GGT CTG CAA GAC TCA GGA CA-3’	5’-AGA GGA AGG GAC TCC ATT G-3’
Prss44/Tessp-4	5’-CAA GGA CAT CAT GGG GAA TA-3’	5’-CTA CCT GCA CCC ACG TTT TA-3’
Gapdh	5’-CAT GGC CTT CCG TGT TCC TA-3’	5’-CCT GCT TCA CCA CCT TCT TGA-3’
Prss/Tessp expression vector		
Prss42/Tessp-2 (*Eco* RI-*Xho* I)	5’-GGA ATT CAA GCC CTG CGA GGC CTG GGC-3’	5’-CCG CTC GAG ACA ATC TGC CTG GTT CAC CA-3’
Prss43/Tessp-3 (*Eco* RV-*Eco* RI)	5’-GGA TAT CAC TTA CAA ACC CAG GGA AGA-3’	5’-GGA ATT CTT AGG GAT CCA TAC GGG AAG CCT-3’
Prss44/Tessp-4 (*Sal* I-*Xho* I)	5’-ACG CGT CGA CAA ATG GGA GAT GCT GAC TTG TG-3’	5’-CCG CTC GAG TTA TTC CAT TTT TGC CAA GC-3’
Marker genes		
rnh2	5’-AGG AAG GAT CTC ATG GAG AG-3’	5’-CTG GCA TAA TCA TGG GTC AG-3’
sycp2	5’-TCC TTG CTT GTC AGC ATT TC-3’	5’-AGA GTG TGG CTC CCA AGA TT-3’
pten2	5’-TTA CCC AGG GAG AAT CTG CT-3’	5’-AGA GTG TTG GTG GAC ATG GA-3’
prm1	5’-ATC AAA ACT CCT GCG TGA GA-3’	5’-AGG TGG CAT TGT TCC TTA GC3’
RT-PCR		
Prss42/Tessp-2	5’-ATG CAT GTC TGT GGA GGT TC-3’	5’-CTG GCA GGC GTC CTT TCC TCT T-3’
Prss43/Tessp-3	5’-AGA TTC CAG GAT CAG GGT CT-3’	5’-GTT ACA GTG CTT CTG GAG GA-3’
Prss44/Tessp-4	5’-GGT CCA CAA GCA GCA CAT CTG-3’	5’-CAG GCA TCT CCT CCT TTC T-3’
Ddx4	5’-TCA GAC GCT CAA CAG GAT GTC C-3’	5’-GGG AGG AAG AAC AGA AGA ACA GGA G-3’
Tes	5’-TGA AGG CAG TGG CTA AAG TGG G-3’	5’-GAG GCT GGT CTG TAA GAG GAG AAA C-3’
Gapdh	5’-CAT GGC CTT CCG TGT TCC TA-3’	5’-CCT GCT TCA CCA CCT TCT TGA-3’

Figure 1

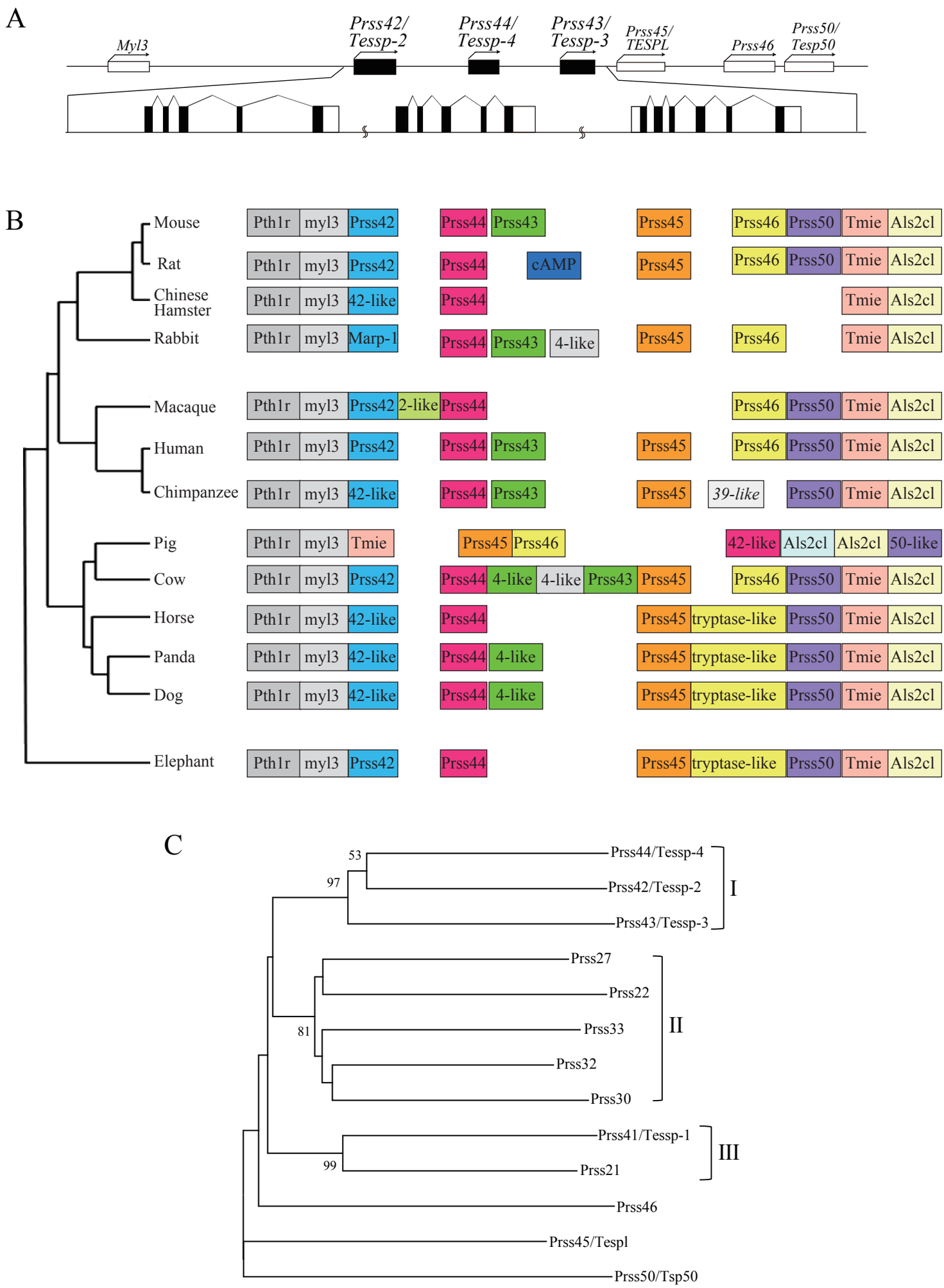
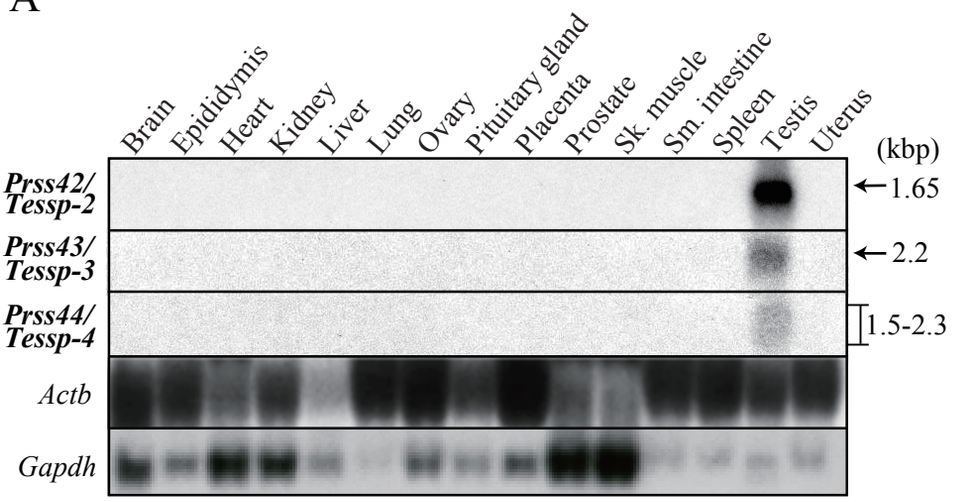

A
Myl3
Prss42/ Tessp-2
Prss44/ Tessp-4
Prss43/ Tessp-3
Prss45/ TESPL
Prss46
Prss50/ Tesp50
B
Mouse
Rat
Chinese Hamster
Rabbit
Macaque
Human
Chimpanzee
Pig
Cow
Horse
Panda
Dog
Elephant
Pth1r
myl3
Prss42
42-like
Marp-1
2-like
Prss44
Prss43
cAMP
4-like
Prss45
Prss46
Prss50
Tmie
Als2cl
39-like
50-like
tryptase-like
C
53
97
81
99
Prss44/Tessp-4
Prss42/Tessp-2
Prss43/Tessp-3
I
Prss27
Prss22
Prss33
Prss32
Prss30
II
Prss41/Tessp-1
Prss21
III
Prss46
Prss45/Tespl
Prss50/Tsp50

Figure 2

A


Brain
Epididymis
Heart
Kidney
Liver
Lung
Ovary
Pituitary gland
Placenta
Prostate
Sk. muscle
Sm. intestine
Spleen
Testis
Uterus
(kbp)
Prss42/ Tessp-2
1.65
Prss43/ Tessp-3
2.2
Prss44/ Tessp-4
1.5-2.3
Actb
Gapdh

B

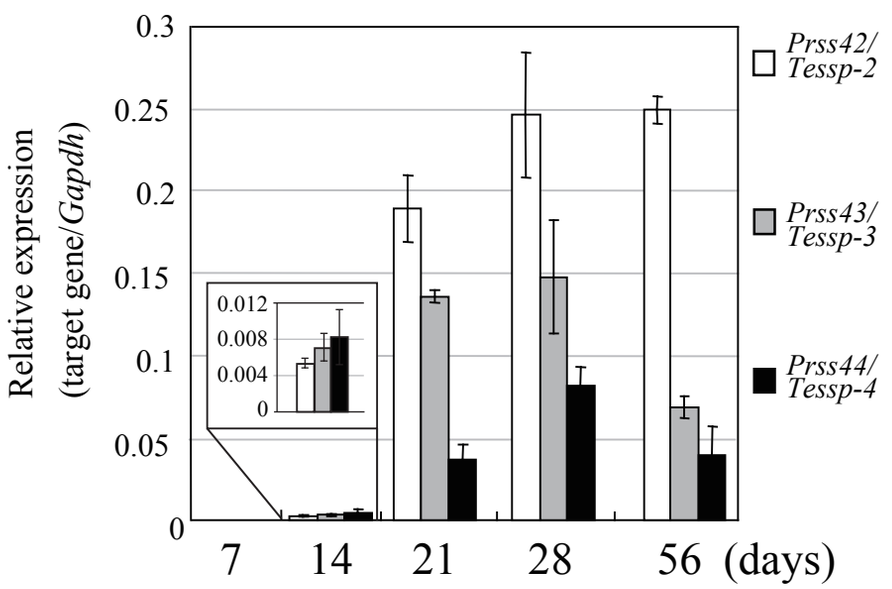

Relative expression
(target gene/Gapdh)
0.3
0.25
0.2
0.15
0.1
0.05
0
0.012
0.008
0.004
0
7
14
21
28
56 (days)
Prss42/ Tessp-2
Prss43/ Tessp-3
Prss44/ Tessp-4

Figure 3

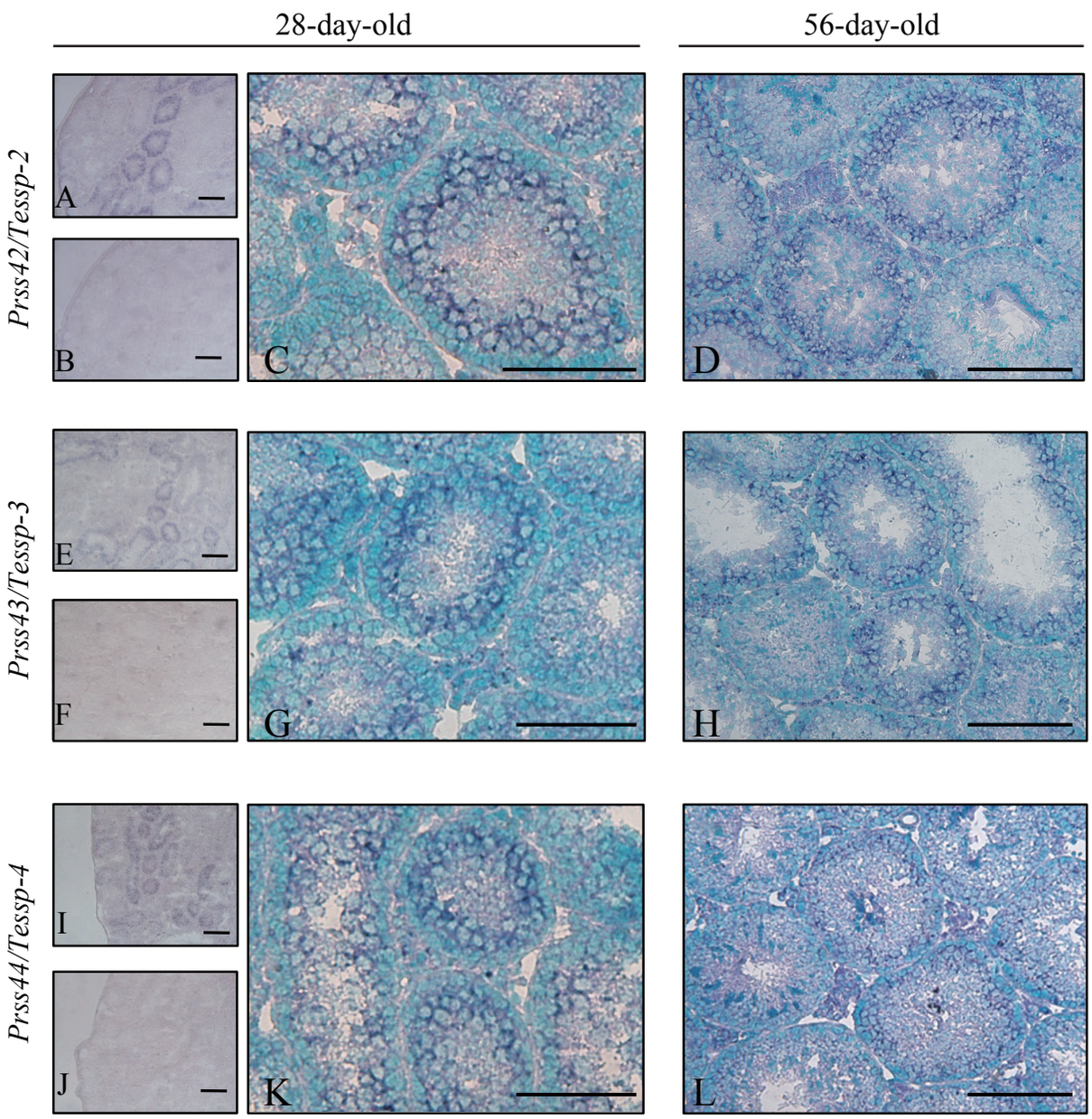

28-day-old
56-day-old
Prss42/Tessp-2
Prss43/Tessp-3
Prss44/Tessp-4
A
B
C
D
E
F
G
H
I
J
K
L

Figure 4

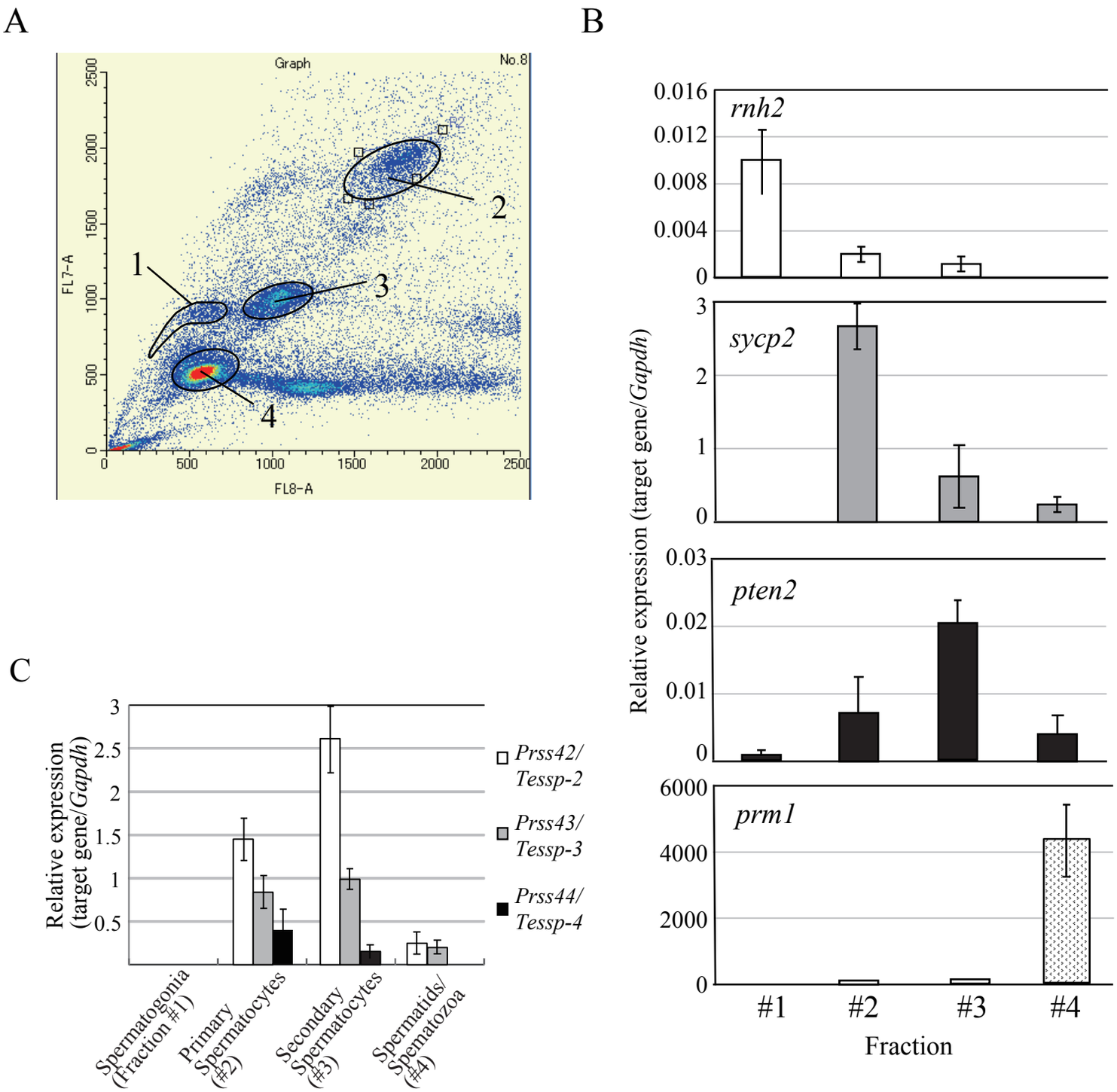

A
Graph
No.8
FL7-A
FL8-A
1
2
3
4
B
rnh2
sycp2
pten2
prm1
Relative expression (target gene/Gapdh)
Fraction
#1
#2
#3
#4
C
Relative expression (target gene/Gapdh)
Prss42/ Tessp-2
Prss43/ Tessp-3
Prss44/ Tessp-4
Spermatogonia (Fraction #1)
Primary Spermatocytes (#2)
Secondary Spermatocytes (#3)
Spermatids/ Spematozoa (#4)

Figure 5

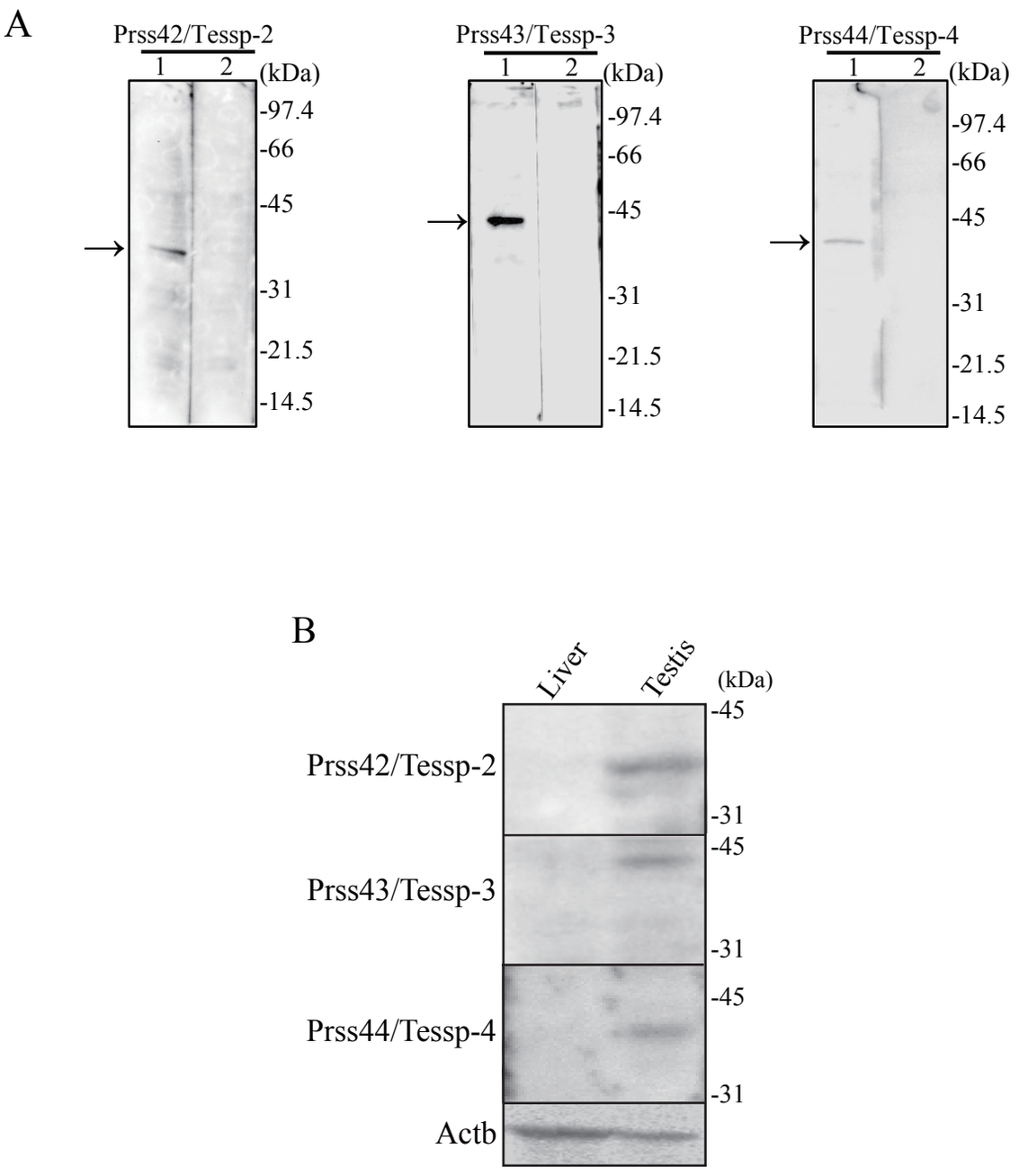
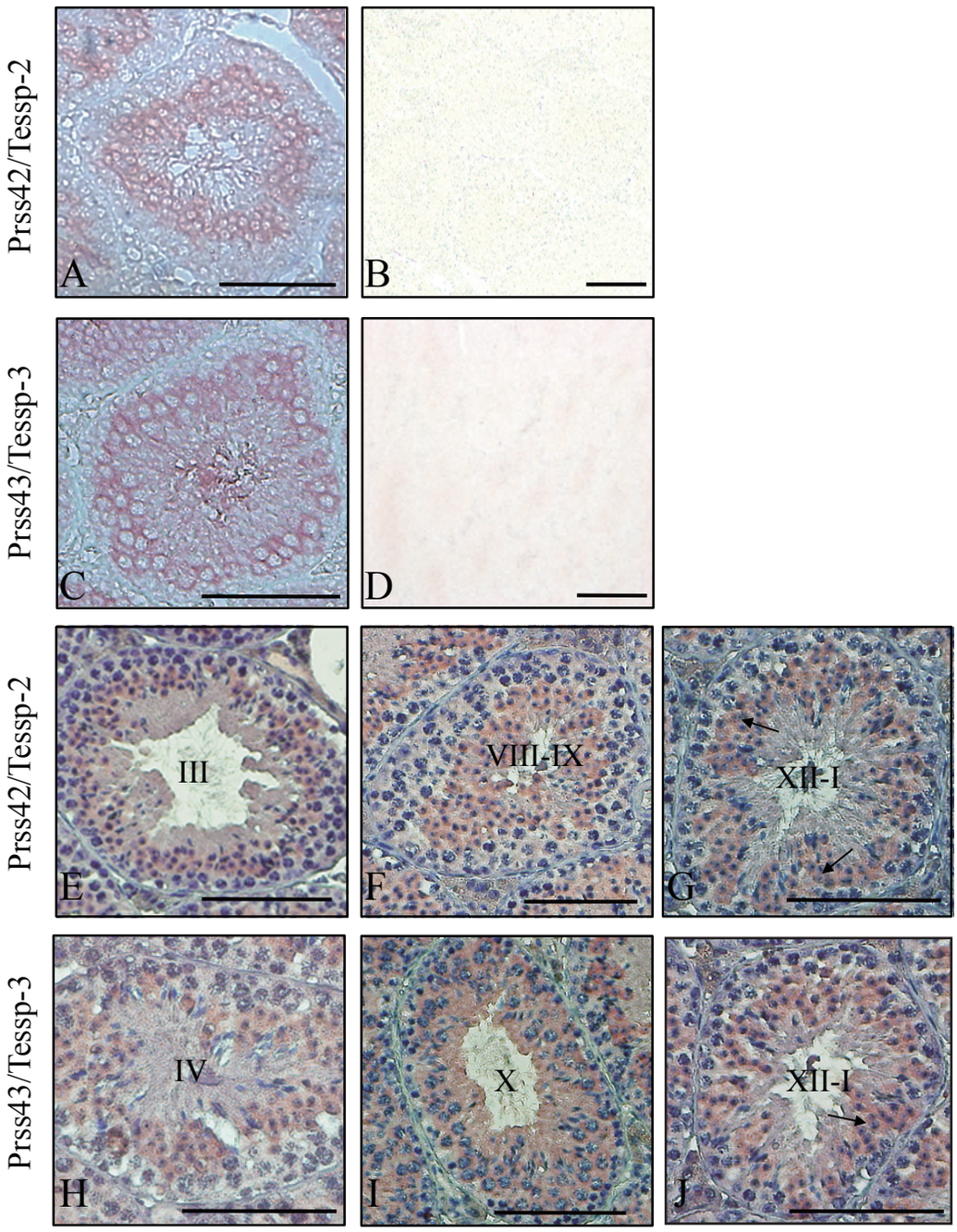

A
Prss42/Tessp-2
1
2
(kDa)
-97.4
-66
-45
-31
-21.5
-14.5
Prss43/Tessp-3
1
2
(kDa)
-97.4
-66
-45
-31
-21.5
-14.5
Prss44/Tessp-4
1
2
(kDa)
-97.4
-66
-45
-31
-21.5
-14.5
B
Liver
Testis
(kDa)
Prss42/Tessp-2
-45
-31
Prss43/Tessp-3
-45
-31
Prss44/Tessp-4
-45
-31
Actb

Figure 6


Prss42/Tessp-2
A
B
Prss43/Tessp-3
C
D
Prss42/Tessp-2
III
E
VIII-IX
F
XII-I
G
Prss43/Tessp-3
IV
H
X
I
XII-I
J

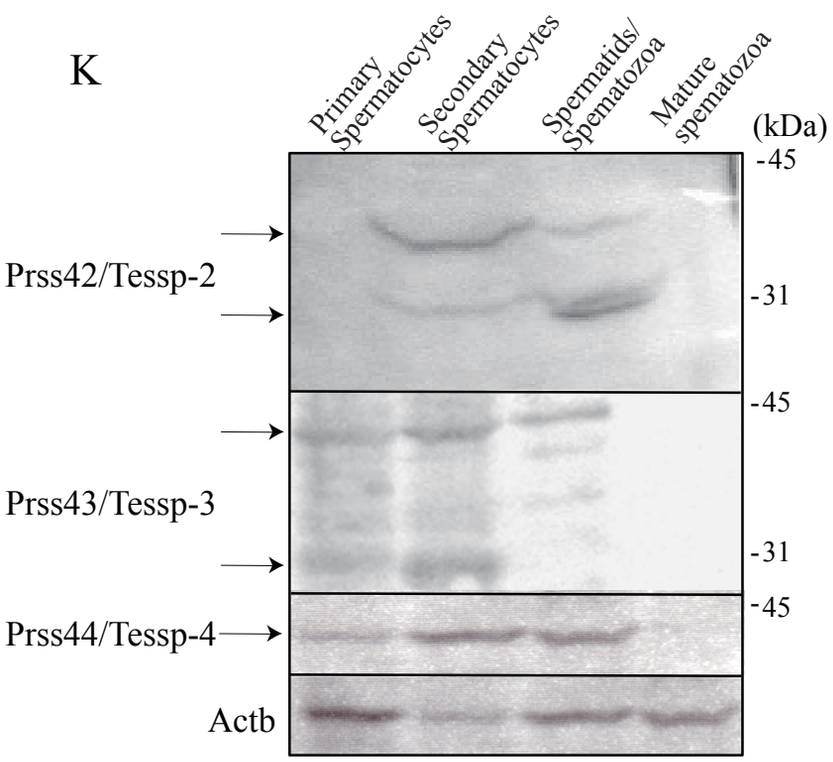

K
Primary Spermatocytes
Secondary Spermatocytes
Spermatids/ Spematozoa
Mature spematozoa
(kDa)
-45
-31
-45
-31
-45
Prss42/Tessp-2
Prss43/Tessp-3
Prss44/Tessp-4
Actb

Figure 7

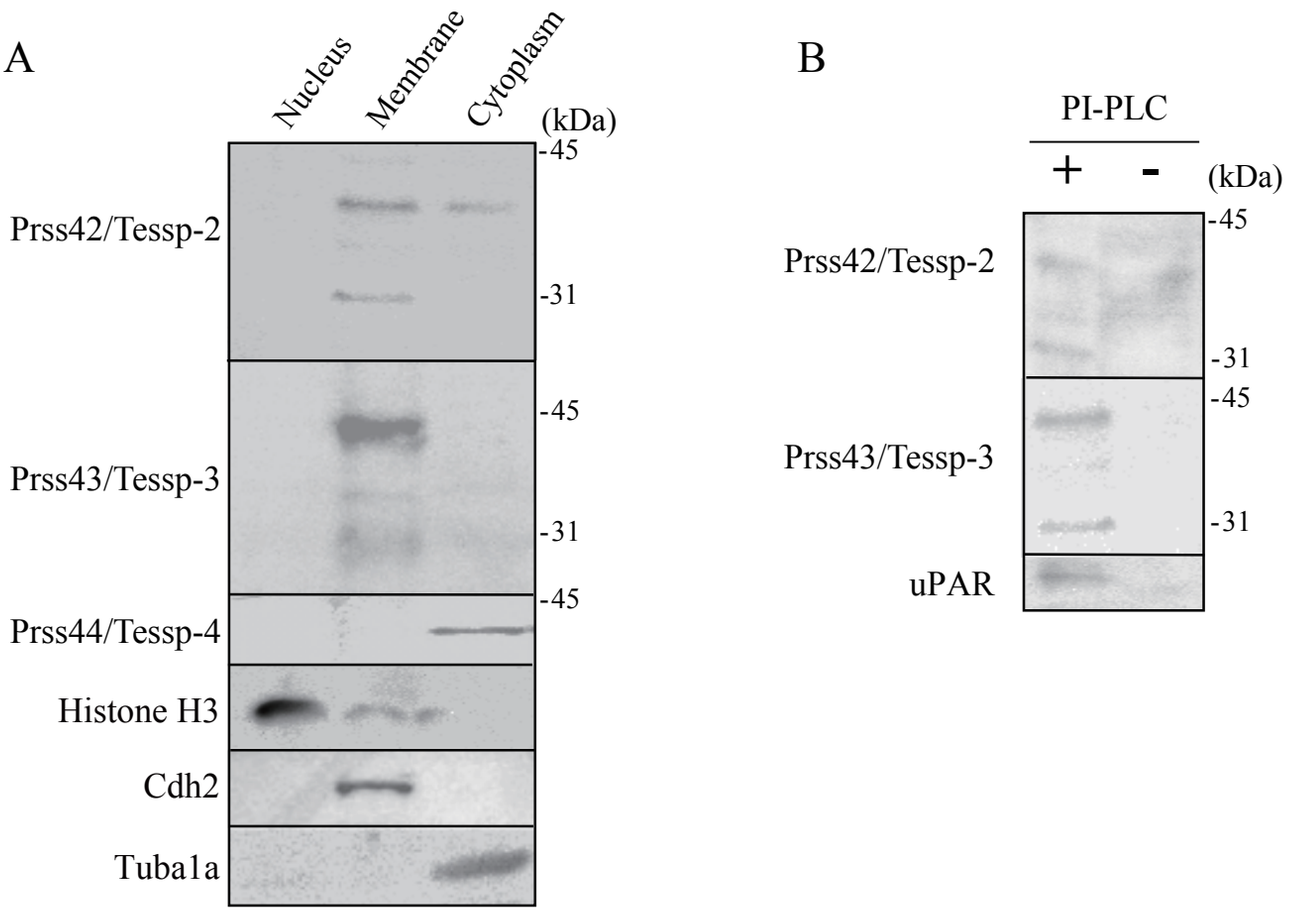
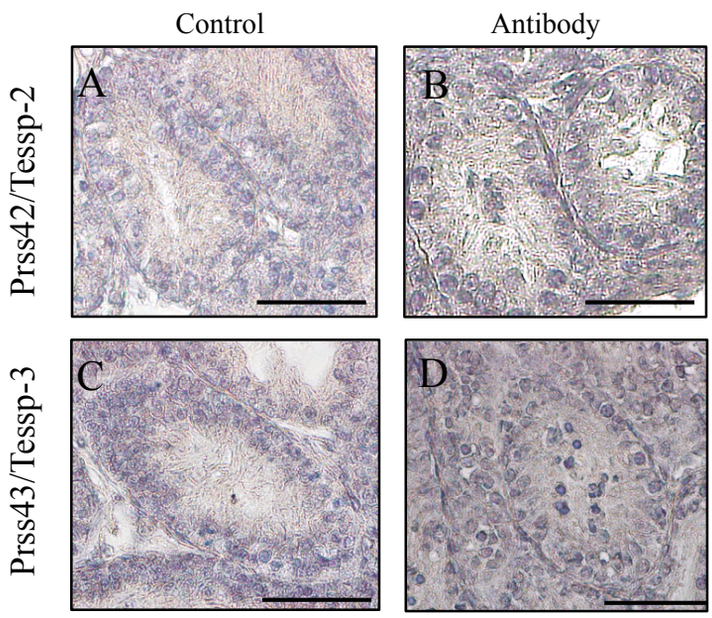
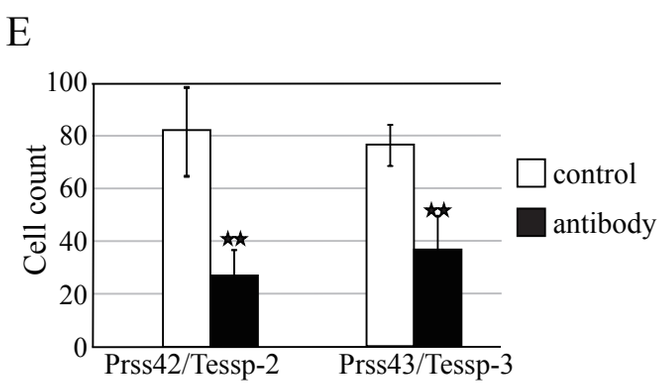
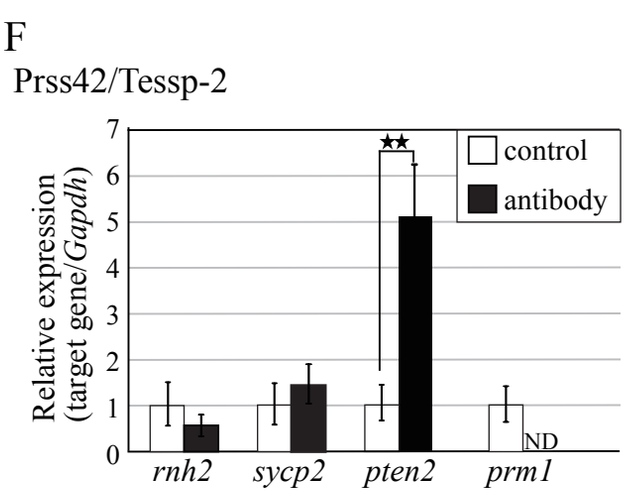

A
Nucleus
Membrane
Cytoplasm
(kDa)
-45
Prss42/Tessp-2
-31
-45
Prss43/Tessp-3
-31
-45
Prss44/Tessp-4
Histone H3
Cdh2
Tuba1a
B
PI-PLC
+
-
(kDa)
-45
Prss42/Tessp-2
-31
-45
Prss43/Tessp-3
-31
uPAR

Figure 8


Control
Antibody
Prss42/Tessp-2
Prss43/Tessp-3
A
B
C
D


E
Cell count
100
80
60
40
20
0
Prss42/Tessp-2
Prss43/Tessp-3
control
antibody


F
Prss42/Tessp-2
Relative expression
(target gene/Gapdh)
7
6
5
4
3
2
1
0
control
antibody
ND
rnh2
sycp2
pten2
prm1

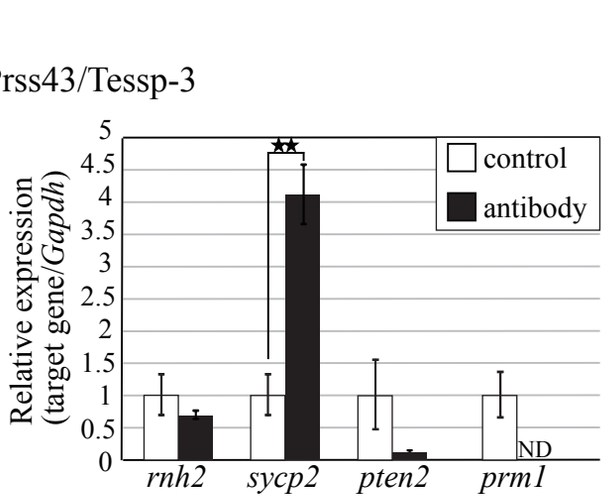
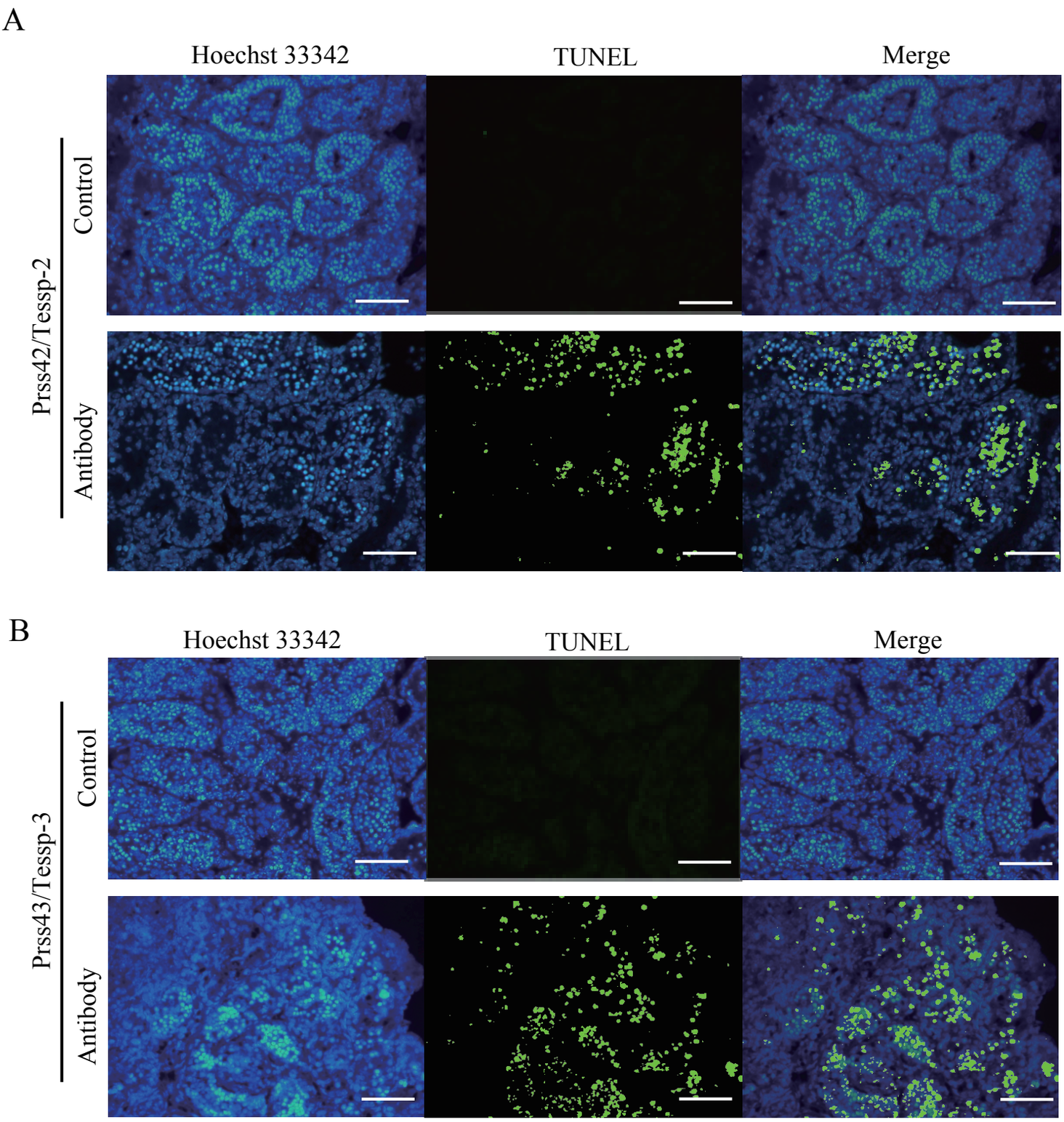

G
Prss43/Tessp-3
Relative expression
(target gene/Gapdh)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
control
antibody
ND
rnh2
sycp2
pten2
prm1

Figure 9


A
Hoechst 33342
TUNEL
Merge
Prss42/Tessp-2
Control
Antibody
B
Hoechst 33342
TUNEL
Merge
Prss43/Tessp-3
Control
Antibody