

# Quantitative Analysis of the Cellular Composition in Seminiferous Tubules in Normal and Genetically Modified Infertile Mice

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## **Abstract**

The aim of this study was to establish a quantitative standard of the cellular composition in seminiferous tubules at each stage of spermatogenesis in the mouse testis, and thereby evaluate abnormalities in the infertile mouse testis. We applied a combination of lectin histochemistry for acrosomes and immunohistochemistry for various specific cell markers, both visualized with fluorescence, on paraffin sections of the testis. We first examined seminiferous tubules from normal mice and counted the number of each cell type at each stage of spermatogenesis. We then examined seminiferous tubules from genetically modified mice deficient (-/-) for one of the cell adhesion molecules, nectin-2 or nectin-3, which were infertile, and compared the number of each cell type at each stage of spermatogenesis with the corresponding value in normal mice. In both nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice, despite the apparently normal morphology of the seminiferous epithelia, the later step spermatids were lost progressively, with the numbers of step 11-16 spermatids in nectin-3<sup>-/-</sup> and step 15-16 spermatids in nectin-2<sup>-/-</sup> mice being significantly lower than those in normal mice. The present study demonstrated that a quantitative analysis of cellular compositions at different stages in seminiferous tubules was useful for evaluating abnormalities in spermatogenesis.

Keywords: spermatogenesis, stage, fluorescence, lectin, immunohistochemistry, cell adhesion molecule

## Introduction

Spermatogenesis is a continuous process from undifferentiated spermatogonia to mature spermatozoa through the mitosis of spermatogonia, meiosis of spermatocytes, and maturation of spermatids that occurs in seminiferous tubules (Russell et al. 1990; Kerr et al. 2006; Hermo et al. 2010). Spermatogenesis has been routinely divided into 12 stages in mice on the basis of changes in the morphology of the acrosome and nucleus using periodic acid-Schiff and hematoxylin (PAS-H) staining (Oakberg 1956; Ahmed and de Rooij 2009; Meistrich and Hess 2013). A section of the mouse testis contains many cross sections of seminiferous tubules, the specific stages of which can be unequivocally determined; however, one tubule section sometimes contains two adjacent stages because of the continuous process of spermatogenesis. Once the stage is determined, all cells present in that section of the seminiferous tubule are potentially identifiable based on the specific combination of different types of spermatogenic cells in each stage (Meistrich et al. 2013).

An earlier study on the mouse testis (Oakberg 1956), using PAS-H staining of 5- $\mu$ m-thick paraffin sections, counted the numbers of type A, intermediate, and type B spermatogonia, spermatocytes in the preleptotene, leptotene, zygotene, pachytene, and diplotene phases of meiosis, and spermatids within individual seminiferous tubules at different stages, but never reported the numbers of Sertoli cells or spermatids in the different steps. Type A spermatogonia have been further classified into several subtypes according to their levels of differentiation, namely, undifferentiated A single ( $A_s$ ), A paired ( $A_{pr}$ ), and A aligned ( $A_{al}$ ) spermatogonia, of which  $A_s$  and  $A_{pr}$  are considered to involve the spermatogenic stem cell population, and differentiated  $A_1$ - $A_4$  spermatogonia (de Rooij et al. 2000; Chiarini-Garcia and Russell 2001). However, distinguishing

these different subtypes of type A spermatogonia requires skillful observations of semi-thin plastic sections with high-resolution light microscopy, and is not applicable to a routine laboratory analysis.

To the best of our knowledge, few studies after that by Oakberg (Oakberg 1956) have performed a quantitative analysis of the cellular composition in mouse seminiferous tubules by counting the numbers of different types of spermatogenic cells in testis sections. The reason for the lack of quantitative studies may be that, with the conventional method using PAS-H staining (Oakberg 1956; Ahmed and de Rooij 2009; Meistrich and Hess 2013) of paraffin sections, it is not necessarily easy to 1) determine the stages of seminiferous tubules precisely because sometimes acrosomal stain with PAS is not conspicuous enough, 2) identify different types of spermatogenic cells based solely on morphological features, or 3) count the numbers of different cell types because excess information from the cytoplasmic stain interferes with nuclear counting. Recent progress in lectin histochemistry and immunohistochemistry (IHC) using fluorescent dye-conjugated molecules appears to have solved such problems. For example, the lectin peanut agglutinin (PNA) is currently being used to visualize the acrosomes of spermatids (Lee and Damjanov 1984; Arya and Vanha-Perttula 1986) and, when combined with nuclear staining with DNA-binding fluorescent dyes such as 4',6-diamidino-2-phenylindole (DAPI), makes it easy and accurate to determine stages and count cell numbers. Furthermore, antibodies against zinc finger and BTB domain containing 16 (ZBTB16) (Buaas et al. 2004; Meistrich and Hess 2013), synaptonemal complex protein 3 (SYCP3) (Lammers et al. 1994), and GATA binding protein 4 (GATA4) (Imai et al. 2004) mainly visualize the nuclei of type A spermatogonia, spermatocytes, and Sertoli cells, respectively, thereby contributing to the precise decision of cell populations in

seminiferous tubules. In the present study, using these fluorescence histochemical techniques, we re-evaluated the cellular composition of seminiferous tubules in a normal mouse testis by counting the numbers of both Sertoli cells and spermatogenic cell types, including spermatids at different steps, in each stage of spermatogenesis.

Many genetically modified and mutant mouse strains with male infertility have recently been reported (de Rooij and de Boer 2003; Matzuk and Lamb 2008). The histological features of the seminiferous epithelia in these mice vary markedly, from the absence of any spermatogenic cells to an apparently normal cell composition. However, such features have been mainly described in a qualitative manner in terms of the shape of spermatogenic cells, and few quantitative analyses have been performed. This led us to perform a quantitative analysis of the cellular composition in each stage of spermatogenesis in the seminiferous tubules of infertile mice and compare it with the standard values obtained in normal control mice. Nectin-2 and nectin-3 are the cell adhesion molecules that are localized in the membranes of Sertoli cells and elongate spermatids, respectively, and undergo heterophilic binding with each other (Ozaki-Kuroda et al. 2002; Takai et al. 2008). In the present study, we analyzed the seminiferous tubules of knockout mice for nectin-2 or nectin-3, in which spermatogenesis is known to progress to the final step, but the nuclei of elongate spermatids are irregular in shape and lead to male infertility (Mueller et al. 2003; Inagaki et al. 2006).

## **Material and Methods**

### **Animals**

The present animal study was specifically approved by Kanazawa University (approval number: AP-132753) and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. C57BL/6 strain male mice (normal mice) were purchased from Nippon SLC, Inc. (Hamamatsu, Japan). Nectin-2-deficient and nectin-3-deficient 129/Sv; C57BL/6 mixed background mice (nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice, respectively) were generated as described previously (Mueller et al. 2003; Inagaki et al. 2006). All animals were reared under standard 12 h light/12 h dark laboratory conditions with free access to standard food and water, and used at the age of 10 weeks. A total of 9 mice (3 for each of control, nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mouse strain) were used in the present study.

### **Tissue Preparation**

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). Saline was initially perfused from the left ventricle to clear the blood, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The testes were then dissected out and further fixed by immersion in the same fixative for 4 h at 4°C. The specimens were dehydrated in a graded ethanol series, embedded in paraffin, cut into 1-, 3-, and 5- $\mu$ m-thick sections using a microtome, and mounted on glass slides. After deparaffinization, the sections were subjected to PAS-H staining, PNA lectin histochemistry, and IHC.



### **PAS-H staining**

Deparaffinized and rehydrated sections were immersed in 0.5% periodic acid solution for 10 min and rinsed in running tap water and several changes of distilled water. They were immersed in Schiff's reagent (Merck, Darmstadt, Germany) for 15 min and then treated with sulfur water (12 mL of sodium bisulfite solution (10%), 10 mL of hydrochloric acid (1 N), and 200 mL distilled water) three times for 3 min each. After rinsing in running tap water and several changes of distilled water, the sections were stained with hematoxylin.

### **PNA lectin histochemistry**

Deparaffinized and rehydrated sections were immersed in 20 mM Tris-HCl buffer (pH 9.0) and heated at 95 °C for 15 min using an autoclave apparatus (ACV-3167N; Asahi Techno Glass, Funabashi, Japan). After cooling to room temperature, the sections were washed in PBS and incubated with the Alexa Fluor 488 conjugate of lectin PNA (1:400; Molecular Probe, Eugene, OR) for 30 min at room temperature to visualize the acrosomes, then counterstained in the nuclei with DAPI (300 nM; Molecular Probe).

### **Antibodies and IHC**

Rabbit anti-ZBTB16 (1:200 dilution; HPA001499, Sigma, St. Louis, MO), rabbit anti-SYCP3 (1:400; ab15093, Abcam, Cambridge, UK), goat anti-GATA4 (1:50; sc-1237, Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-Nectin-2 (1:100; ab16912, Abcam), and rabbit anti-nectin-3 (1:100; ab63931, Abcam) polyclonal antibodies were used. Deparaffinized and rehydrated sections were immersed in 20 mM Tris-HCl buffer (pH 9.0) and heated at 95 °C for 15

min using the autoclave apparatus to retrieve the antigens. After cooling to room temperature, the sections were washed in PBS and treated with 1% skim milk/PBS for 1 h at room temperature to prevent non-specific antibody binding and then incubated overnight at 4°C with the primary antibodies. After washing in PBS, the sections were incubated with the appropriate fluorescent dye-conjugated secondary antibody (Alexa Fluor 594- or 488-conjugated donkey anti-rabbit, -rat, or -goat IgG antibodies; 1:400; Molecular Probe) for 1 h at room temperature. In double IHC, the sections were incubated with the mixture of goat anti-GATA4 and rabbit anti-ZBTB16 or anti-SYCP3 primary antibodies followed by a mixture of fluorescent anti-goat and anti-rabbit IgG secondary antibodies. In the combination of lectin histochemistry and IHC, sections were first treated with IHC for cell markers and then with PNA lectin histochemistry for acrosomes. The sections were then counterstained in the nuclei with DAPI, mounted on glass slides with Fluoromount (Diagnostic BioSystems, Pleasanton, CA), and examined with a fluorescence microscope (BX50/BX-FLA; Olympus, Tokyo, Japan) using filters passing blue, green and red emissions. Where necessary, Fluoromount was removed and the sections were washed and stained with hematoxylin and eosin (HE).

### **Cell quantification procedures**

Cell quantification studies were performed to obtain the absolute numbers of different cell types per section of seminiferous tubule at each stage. In every single testis from normal, nectin 2<sup>-/-</sup>, or nectin 3<sup>-/-</sup> mice, each 3 in number, 2 portions separated by a distance of more than 100 μm were selected, and two 3-μm-thick serial sections were cut at each portion. One of the serial sections from each portion was double-immunostained for GATA4 and ZBTB16, and the other for GATA4

and SYCP3. Thereafter, all 4 sections in every testis were stained with PNA-lectin histochemistry and DAPI, and observed at the 400-fold total magnification using the 40-fold objective lens with the numerical aperture 0.85. In all sections, the seminiferous tubules that were cut in orthogonal transversal sections were selected, and the images obtained at different wavelengths were overlapped using Photoshop CS6 software (Adobe Systems, Inc., Mountain View, CA). In all seminiferous tubules, the stages of spermatogenesis were determined by the shape of acrosomes according to the known criteria (Oakberg 1956; Ahmed et al. 2009; Meistrich et al. 2013), and the different cell types were identified based on the shape and size of nuclei as well as chromatin patterns and marker immunoreactivity in the nuclei. Among the 2 pairs of serial sections of seminiferous tubules, the sections immunostained for ZBTB16 were used to analyze stages I-VI, and those immunostained for SYCP3 were used to analyze stages VII-XII. The numbers of Sertoli cells and all types of spermatogenic cells per seminiferous tubule were counted using ImageJ software (<http://imagej.nih.gov/ij/>).

### **Statistical analysis**

The numbers of specific cell types per seminiferous tubule section were presented as the mean  $\pm$  standard deviation (SD). A comparison of values among different stages within a single testis from a representative normal mouse was performed with a one-way analysis of variance (ANOVA) followed by Scheffe's F test, and differences with  $P < 0.01$  were considered significant. A comparison of values in the same stage among testes from control, nectin-2<sup>-/-</sup>, and nectin-3<sup>-/-</sup> mice, each 3 in number, was performed with a one-way ANOVA followed by Bonferroni's posthoc test, and differences with  $P < 0.05$  were considered significant.

## **Results**

### **Optimal section thickness**

To determine the optimal conditions for the quantitative analysis, we prepared serial sections of the normal mouse testis with different thicknesses of 1, 3 and 5  $\mu\text{m}$ , stained their nuclei with DAPI, and compared images of the same single seminiferous tubule at 400-fold magnification (Fig. 1A-C). In the 1- $\mu\text{m}$  and 3- $\mu\text{m}$ -thick sections, almost all cell nuclei were in focus, whereas some of the nuclei of elongate spermatids in the 5- $\mu\text{m}$ -thick sections were out of focus and difficult to recognize, suggesting that the nuclei of elongate spermatids were small enough to be located at various depths within the sections. On the other hand, it was difficult to determine whether to count some of the larger nuclei of spermatocytes in the 1- $\mu\text{m}$ -thick sections because of their very small size, suggesting that they may represent the nuclei cut at the edges. Taking into consideration that the calculated depth of focus for present observation was  $\pm 1.20 \mu\text{m}$  (for blue fluorescence), and that consistently preparing 1- $\mu\text{m}$ -thick paraffin sections was technically difficult, we adopted 3  $\mu\text{m}$  as the thickness of sections and observed them at 400-fold magnification in subsequent quantitative analyses.

### **Determination of stages in seminiferous tubules**

Using only DAPI staining of nuclei, it was possible to roughly distinguish different cell populations in the seminiferous tubules based on the position, size, and shape of nuclei as well as the appearance of chromatin in the nuclei, and thereby determine the stages of seminiferous tubules

based on the patterns of combinations of these cell populations (Meistrich and Hess, 2013). Determining the stages became markedly easier and more reliable with acrosome staining of spermatids. In the present study, the acrosomes of spermatids at various steps were visualized with PNA lectin histochemistry in the same patterns as in those visualized with PAS-H staining, but more conspicuously than the latter (Fig. 1D, E). The 12 stages of spermatogenesis were then easily determined using the combination of PNA lectin histochemistry for acrosomes and DAPI staining for chromatin in the nuclei, according to the established criteria of stages based on the position, size, and shape of acrosomes (Fig. 2). Once the stages were determined, the identification of the cell populations became easier because the patterns of combinations of different types of spermatogenic cells in a particular stage had been established (Oakberg 1956; Ahmed and de Rooij, 2009; Meistrich and Hess 2013). For example, intermediate spermatogonia only existed in stages II-IV and type B spermatogonia in stages V and VI, whereas type A spermatogonia existed in almost all stages. They were distinguishable from each other due to the size and shape of the nuclei and the pattern of the nucleolus and heterochromatin in the nuclei. However, it was sometimes difficult to accurately distinguish the former from the latter in stages in which type A spermatogonia co-existed with intermediate or type B spermatogonia. Although the round and elongating spermatids (steps 1-12) were distinguishable from co-existing spermatocytes with the presence of PNA lectin-stained acrosomes, it was sometimes difficult to distinguish among the earlier spermatocytes, spermatogonia, and Sertoli cells, which co-existed in contact with the basement membrane, based solely on the size, shape, and position of the nuclei and patterns of the nucleoli and condensed chromatin in the nuclei.

## **Identification of cell populations in seminiferous tubules**

To simplify the identification of cell populations and make it more reliable, we performed IHC using antibodies against specific cell markers and examined the immunopositive cell types known to be present in each stage of spermatogenesis. We confirmed that immunoreactivities for ZBTB16, SYCP3, and GATA4 were generally localized in the nuclei of type A spermatogonia, spermatocytes, and Sertoli cells, respectively, by comparing fluorescence images with HE-stained images (Fig. 3). We further analyzed sections of seminiferous tubules in different stages of spermatogenesis that were double-immunostained for either GATA4 and ZBTB16 or GATA4 and SYCP3, in combination with PNA lectin histochemistry for acrosomes and DAPI staining for nuclei (Fig. 4, 5). Although both PNA and anti-GATA4 antibody were conjugated with Alexa Fluor 488 that yields the same green fluorescence, there was no possibility of their crossing reaction, because PNA did not stain Sertoli cell nuclei (Fig. 2), and anti-GATA4 antibody did not stain spermatid acrosomes (Fig. 3). ZBTB16 was localized in the nuclei of type A spermatogonia in all stages and weakly in intermediate spermatogonia in stage II-III, but not in IV (Fig. 4). SYCP3 was localized in the nuclei of preleptotene spermatocytes in stage VIII, but not in VII, leptotene spermatocytes in stages IX and X, zygotene spermatocytes in stages XI and XII, pachytene spermatocytes in stages I through X, diplotene spermatocytes in stage XI, meiotic cells (subsequently defined as cells undergoing the first and second meiotic divisions, including secondary spermatocytes) in stage XII, and weakly in step 1 spermatids in stage I (Fig. 5). GATA4 was localized in the nuclei of Sertoli cells in all stages (Fig. 4, 5). However, even with the help of IHC for the marker proteins, it was difficult to distinguish the different subtypes of type A spermatogonia (de Rooij and Russell 2000; Chiarini-Garcia and Russell 2001). The use of strong fixatives such as Bouin or glutaraldehyde and

embedding in plastic resins were previously reported to be necessary for this purpose, which makes it difficult to apply IHC to these sections. Therefore, in the present study we did not attempt to distinguish the subtypes of type A spermatogonia. Figure 6 summarized the patterns of acrosomal staining with PNA lectin and nuclear staining for ZBTB16, SYCP3, and GATA4 at different stages of spermatogenesis based on the results of Figures 4 and 5. As shown in the schematic, we found that ZBTB16 staining was useful for distinguishing stages I, II-III, and IV, whereas SYCP3 staining was useful for distinguishing stages VII, VIII, and IX.

### **Quantification of the cell composition in normal mouse seminiferous tubules**

To perform a quantitative analysis of the cell composition in seminiferous tubules, we applied combinations of DAPI staining, PNA lectin histochemistry, and IHC for specific cell markers. In a total of 322 seminiferous tubules, i.e., 157 and 165 from 2 separate portions, in the representative normal mouse testis, the stages were determined by the shape of the acrosomes, and the cell types in each tubule were identified based on the position, size, and shape of nuclei, the appearance of the nucleolus and chromatin in the nuclei, and specific marker staining in the nuclei. Of the 2 serial sections in each portion of the testis, one double-immunostained for GATA4 and ZBTB16 and the other for GATA4 and SYCP3, the former was used to analyze stages I-VI and the latter to analyze stages VII-XII. The numbers of Sertoli cells, type A, intermediate, and type B spermatogonia, as well as preleptotene, leptotene, zygotene, pachytene, diplotene spermatocytes, and meiotic cells, and each of step 1 through to 16 spermatids were counted at each stage in the seminiferous tubule (Table 1). The average number of Sertoli cells per tubule section was  $18.1 \pm 3.4$  in all seminiferous tubules examined ( $n = 322$ ), and was not significantly different between all stages. Therefore, in

subsequent quantitative analyses, the number of each type of spermatogenic cell was counted as the absolute number per tubule section, without normalization with the number of Sertoli cells in the same section. The number of type A spermatogonia remained low from stages II-III through to VIII, increased gradually from IX through XII, and was the largest in stage I. Intermediate spermatogonia were exclusively detected in stages II-IV with an average number of  $11.8 \pm 4.1$  per tubule section, while type B spermatogonia were exclusively detected in stages V-VI, with the average number being  $26.1 \pm 6.3$  per tubule section. No significant differences were observed in the numbers of preleptotene spermatocytes in stages VII and VIII, leptotene spermatocytes in stages IX and X, pachytene spermatocytes from stage I through to X, and diplotene spermatocytes in stage XI between the stages, with the overall average being  $51.4 \pm 8.7$  per tubule section. Meiotic cells were exclusively detected in stage XII, with the average number being  $96.3 \pm 31.3$  per tubule section. The average number of round and elongating spermatids (steps 1-12) through all seminiferous tubules was  $144.6 \pm 29.8$  and did not significantly differ between the stages. On the other hand, the average number of elongated spermatids (steps 13-16) present in stages I-VIII was, for the most part, significantly lower than that of round spermatids (steps 1-8) present in the same respective stages, and varied from stage to stage.

### **Morphological features of infertile mouse seminiferous tubules**

In the testes from both nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice stained with PAS-H, the basic structure of the seminiferous tubules was not severely defective, and it was possible to identify 12 stages of spermatogenesis as in the control mouse testis based on the acrosomal patterns in round and elongating spermatids (steps 1-12). However, the nuclei of elongated spermatids (steps 13-16),



which are present in stages I-VIII of spermatogenesis, appeared to be more irregular in shape and fewer in number than those in the corresponding stages in normal control mice (Fig. 7A, B, E, F, I, J). This was more prominent in nectin-3<sup>-/-</sup> than in nectin-2<sup>-/-</sup> mouse testes, with step 16 spermatids being rarely detected in stage VII and VIII seminiferous tubules in the former (Fig. 7J). Other types of spermatogenic cells were normal in appearance. IHC indicated that immunoreactivity for nectin-2 was localized in the membranes of Sertoli cells in control and nectin-3<sup>-/-</sup> mice, but was completely absent in nectin-2<sup>-/-</sup> mice (Fig. 7C, G, K). On the other hand, immunoreactivity for nectin-3 was localized in the membranes surrounding the head portion of elongate (step 8 and later) spermatids in control and nectin-2<sup>-/-</sup> mice, but was completely absent in nectin-3<sup>-/-</sup> mice (Fig. 7D, H, L). Furthermore, immunoreactivity for nectin-2 was detected only in the basal portions, but not in the apical portions of Sertoli cell membranes in nectin-3<sup>-/-</sup> mice (Fig. 7K).

### **Quantification of the cell composition in infertile mouse seminiferous tubules**

As in control mice, we performed a quantitative analysis of the cellular composition of seminiferous tubules in nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice using a combination of PNA lectin histochemistry for the acrosomes, DAPI staining in the nuclei, and IHC for SYCP3, ZBTB, and GATA4. Using SYCP3 immunostaining, which enabled stages VII, VIII, and IX to be distinguished even in the absence of elongated spermatids in nectin-3<sup>-/-</sup> mice (Fig. 7M-O), we were able to determine all stages of spermatogenesis in nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice as in control mice. The numbers of all cell types in each stage were counted in a total of 197 seminiferous tubules, i.e., 100 and 97 from 2 separate portions, in a representative testis of nectin-2<sup>-/-</sup> mice, and 220 (108 and 112) in a representative testis of nectin-3<sup>-/-</sup> mice, and were then compared with the values

obtained from the same representative testis of control mice shown in Table 1 (Fig. 8). The average numbers of Sertoli cells per tubule section were not significantly different between the stages and were  $17.0 \pm 3.2$  ( $n = 197$ ) and  $16.8 \pm 3.5$  ( $n = 220$ ) in all seminiferous tubules examined in nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice, respectively, which were similar to  $18.1 \pm 3.4$  ( $n = 322$ ) in control mice. No apparent differences were observed in the average numbers of type A, intermediate, and type B spermatogonia, as well as preleptotene, leptotene, zygotene, pachytene, diplotene spermatocytes, and meiotic cells among the 3 mouse strains. However, as was noted in the morphological analysis, the number of elongated spermatids (steps 13-16) appeared to become progressively lower in nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice than in control mice, starting from earlier stages in nectin-3<sup>-/-</sup> than in nectin-2<sup>-/-</sup> mice. Therefore, we performed a statistical analysis of differences in the average numbers of step 10 and later spermatids per tubule section starting from stage X between control and nectin-2<sup>-/-</sup> or nectin-3<sup>-/-</sup> mice using 3 animals each (Fig. 9). We confirmed that the numbers of step 15-16 spermatids in nectin-2<sup>-/-</sup> mice and step 11-16 spermatids in nectin-3<sup>-/-</sup> mice were significantly lower than the numbers of corresponding spermatids in control mice ( $P < 0.05$ ,  $n = 3$ ). At stage VIII, the numbers of matured step 16 spermatids in nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice were  $43.2 \pm 3.4$  and  $4.4 \pm 1.4$ , respectively, which were 45% and 5%, respectively, of  $96.3 \pm 4.4$  in control mice ( $P < 0.05$ ,  $n = 3$ ).

## **Discussion**

In the present study, we first aimed to establish a quantitative standard for the cellular composition of seminiferous tubules in normal adult mice using fluorescence histochemical techniques. Determining the stages of seminiferous tubules and counting the cell number at each stage was easier with the combination of PNA lectin histochemistry for acrosomes and DAPI staining for nuclei than with the PAS-H method. The PAS-H method is based on the purple-red-colored PAS staining of acrosomal glycoproteins and the violet-colored hematoxylin staining of nucleic acids (Ahmed et al. 2009). As spermiogenesis proceeds, the acrosome derived from the Golgi apparatus increases in size and spreads as a flat cap over the nucleus, which undergoes shrinkage due to the condensation of chromatin. The shape of acrosomes in spermatids in various steps, as well as the combination pattern of various types of spermatogenic cells in the seminiferous epithelium, make it possible to determine the stage of spermatogenesis in a given section of a seminiferous tubule (Meistrich and Hess 2013). Spermatogenesis has been classified into 14 stages in rats (Leblond and Clermont 1952; Russell et al. 1990) and into 12 stages in mice by the PAS-H method (Oakberg 1956; Ahmed and de Rooij 2009; Meistrich and Hess 2013). In the present study, fluorescence-labeled acrosomes and nuclei were easier to recognize than PAS-H-stained ones because the green fluorescence of acrosomes was a good contrast to the blue fluorescence of nuclei, both of which were a good contrast to the fluorescence-free background of the cytoplasm. The greatest advantage of the fluorescence method is that multiple antigens can be visualized with IHC in combination with acrosomes visualized with lectin histochemistry. In the present study, using IHC for ZBTB16, SYCP3, and GATA4, specific nuclear markers for type A

spermatogonia, spermatocytes, and Sertoli cells, respectively, we easily and precisely identified the different cell types in each stage of the seminiferous tubules.

The present results on the quantitative composition of spermatogenic cells in the seminiferous tubules of normal adult mice at different stages were consistent with the earlier findings of Oakberg (Oakberg 1956); however, we showed the numbers of Sertoli cells and spermatids at all different steps while Oakberg did not. In the present study, the average numbers of intermediate spermatogonia, type B spermatogonia, and spermatocytes (preleptotene-diplotene) per tubule in all tubule sections that contain the respective cell types were  $11.8 \pm 4.1$ ,  $26.1 \pm 6.3$ ,  $51.4 \pm 8.7$ , respectively, which were roughly 1:2:4 in proportion, in agreement with the theoretical values deduced from the final two mitotic divisions of differentiated spermatogonia. On the other hand, the average number of step 1-12 spermatids was  $144.6 \pm 29.8$ . This number was approximately 2.81-fold higher than that of spermatocytes, which was only 70% of the theoretical value 4.0 deduced from two sequential meiotic divisions of spermatocytes.

However, the ratio of the numbers of spermatids to spermatocytes within a section needs to be corrected with the probability of cells to be involved in the cross sections of a testis, which is a function of the diameter of cells, the distance between neighboring cells, and the thickness of the sections (Abercrombie 1946; Oakberg 1956). Therefore, we randomly selected 10 tubules from each of 9 stages (I, II-III through to X) and measured the nuclear diameter of one typical pachytene spermatocyte in each tubule at each stage. The average nuclear diameter of pachytene spermatocytes at each stage ( $n = 10$ ) ranged between  $9.165 \mu\text{m}$  (at stage I) and  $11.520 \mu\text{m}$  (at stage X). The corrected count (CC) of pachytene spermatocytes per tubule at each stage was calculated using following equation, which assumed that the nuclear distance was equal to the section

thickness (Abercrombie 1946; Oakberg 1956):  $CC = \text{number of pachytene spermatocytes} \times \text{section thickness (3 } \mu\text{m)} / (\text{section thickness} + \text{nuclear diameter})$ . The calculated mean CC of pachytene spermatocytes per tubule in all stages ( $n = 9$ ) was 12.165. We also measured the nuclear diameter of one typical step 2-3 spermatid in each of the randomly selected 10 tubules at stage II-III, and determined the average nuclear diameter of step 2-3 spermatids ( $n = 10$ ) to be 6.362  $\mu\text{m}$ . The calculated CC of step 2-3 spermatids per tubule at stage II-III was 47.996. Therefore, the corrected ratio of the numbers of step 2-3 spermatids to pachytene spermatocytes was  $47.996 / 12.165 = 3.945$ . This was 98.6% of the theoretical value of 4.0. This calculation could be roughly applied to the ratio of all step 1-12 spermatids to all spermatocytes. Together with no significant difference being observed in the average numbers of preleptotene, leptotene, zygotene, and pachytene spermatocytes, the present results implied that the loss of cell numbers, if any, was very small during the meiosis of spermatocytes.

The average numbers of elongated (step 13-16) spermatids at stages I-VIII ranged between  $95.4 \pm 17.2$  and  $125.5 \pm 23.2$ , which were mostly lower than those of round spermatids (steps 1-8) present in the respective stages, and varied from stage to stage. However, the ratio of elongated to round spermatids may also need to be corrected because 1) some of the nuclei of elongated spermatids, which were very small, may have been out of the focus and not counted in the 3- $\mu\text{m}$ -thick sections, and 2) the expected numbers of nuclei in elongated spermatids in sections may have been less than those in round spermatids due to differences in their diameters. Therefore, the loss in cell numbers, if any, may have been small during the process of spermiogenesis. Large numbers of spermatogenic cells are thought to undergo apoptotic death in the testis (Oakberg 1956; Hess et al. 2008). However, the present results implied that such apoptosis may mainly occur in

earlier (type A) spermatogonia, but not in other cell types. It is important to note that the present analysis was only performed in random portions of seminiferous tubules included in a 3- $\mu$ m section of the testis. To achieve a more precise analysis of changes in the cellular composition accompanying spermatogenesis, cell numbers in 3D-reconstructed seminiferous tubules should be evaluated in future.

In the present study, based on the quantitative standard of the cell composition in the seminiferous tubules obtained in normal control mice, we revealed a progressive decrease in the numbers of later step spermatids in the seminiferous tubules of genetically modified mice deficient (-/-) in nectin-2 or nectin-3, with the numbers of steps 11-16 in nectin-3<sup>-/-</sup> and steps 15-16 in nectin-2<sup>-/-</sup> mice being significantly lower than those in control mice. Nectin-2 and nectin-3 are Ca<sup>2+</sup>-independent immunoglobulin-like cell adhesion molecules (Takai et al. 2008). In the mouse testis, nectin-2 and nectin-3 are localized in the membranes of Sertoli cells and elongate (step 8-16) spermatids, respectively, and undergo heterophilic binding with each other at the junctions between these cell types (Ozaki-Kuroda et al. 2002). Knockout mice for nectin-2 (nectin-2<sup>-/-</sup>) have male-specific infertility due to severe spermatozoon head and midpiece malformation, impaired binding to the zona pellucida, and oocyte penetration (Mueller et al. 2003). Knockout mice for nectin-3 (nectin-3<sup>-/-</sup>) also have male-specific infertility and exhibit defects in the later steps of sperm morphogenesis including distorted nuclei and the abnormal distribution of mitochondrias, as well as the impaired localization of nectin-2 at the Sertoli–spermatid junctions (Inagaki et al. 2006). These suggest that the heterophilic trans interaction between nectin-2 and nectin-3 may be essential for the formation and maintenance of Sertoli–spermatid junctions, which may play a critical role in spermatid development. The present study performed a

quantitative analysis of spermatogenic cells in the seminiferous tubules of nectin-2<sup>-/-</sup> and nectin-3<sup>-/-</sup> mice for the first time. The results obtained were consistent with the proposed roles of nectin-2 and nectin-3 in the final phase of spermatogenesis, i.e., spermiogenesis. The absence of the interaction of elongate spermatids with Sertoli cells through binding of these cell adhesion molecules may cause impairments in the maturation of these spermatids and lead to their progressive detachment from Sertoli cells. The present study also revealed that decreases in the numbers of spermatids began from the earlier steps and were more severe in nectin-3<sup>-/-</sup> than in nectin-2<sup>-/-</sup> mice. This may be interpreted as the lack of nectin-2 in the apical portions of Sertoli cell membranes in nectin-3<sup>-/-</sup> mice, a phenomenon that was reported previously by Inagaki et al. (2006) and confirmed in the present study. In the case of nectin-2<sup>-/-</sup> mice, intact nectin-3 on later step spermatids may bind with some cell adhesion molecules other than nectin-2 on Sertoli cells, which may compensate for the absence of nectin-2.

Various natural mutants as well as genetically modified mouse strains have male-specific infertility (de Rooij and de Boer 2003; Matzuk and Lamb 2008). In some of these infertile strains, the histological features of seminiferous epithelia appear normal, or not severely impaired. However, the application of a quantitative analysis to these strains may disclose specific points of the abnormality in spermatogenesis, as was accomplished in the present study. Thus, the present procedure can become a standard in the quantitative analysis of abnormalities in spermatogenesis. The ultimate goal of the present study is the application of this procedure to the study of human male infertility. Most cases of male infertility have been attributed to impaired spermatogenesis (non-obstructive azoospermia), which is mostly idiopathic (Lipshultz et al. 2009). The state of spermatogenesis in the seminiferous tubules of infertile males has been evaluated using the

Johnsen score, a testicular biopsy score that covers scores from 1 (neither germ cells nor Sertoli cells present) through to 10 (complete spermatogenesis and perfect tubules). However, this evaluation method is largely qualitative and does not distinguish spermatogenic stages; therefore, cases in which spermatogenesis normally progresses to the later steps, but ultimately leads to complete infertility, can be apparently assessed as higher scores. The difficulty associated with the quantitative evaluation of spermatogenesis in human seminiferous tubules resides in the area of each stage being so small that a single section of seminiferous tubules commonly contains several different stages, as well as the acrosomes of spermatids being difficult to stain with the conventional PAS-H, such that human spermatogenesis is only divided into 6 stages based solely on cell associations (Clermont 1963). However, a recent study reported that the visualization of acrosomes with IHC for acrosin enabled 12 stages to be distinguished in human spermatogenesis (Muciaccia et al. 2013). Thus, providing that a precise determination of stages is achieved, the present quantitative procedure will become useful in disclosing the specific points of abnormalities in spermatogenesis with human infertility.



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**Declaration of Conflicting Interests**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Figure and Table Legends

Figure 1. A-C, sections of the seminiferous epithelium (presumably stage VII or VIII) 1  $\mu\text{m}$  (A), 3  $\mu\text{m}$  (B) and 5  $\mu\text{m}$  (C) in thickness stained in the nuclei with DAPI. D, E, stage VIII seminiferous tubules shown in fluorescence (D) and PAS-H staining (E). Acrosomes were stained with PNA lectin histochemistry (green; D) or PAS (E), and nuclei were stained with DAPI (blue; D) or hematoxylin (E). Bars = 15  $\mu\text{m}$  (A-C), 30  $\mu\text{m}$  (D, E).

Figure 2. Determination of stages (I-XII) and identification of cell types. Three- $\mu\text{m}$  thick sections were stained in the nuclei with DAPI (blue) and in the acrosomes with PNA lectin histochemistry (green). Se, Sertoli cells; A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M1, spermatocytes undergoing the first meiotic division; Sc, secondary spermatocytes; S1-16, step 1-16 spermatids. Bar = 10  $\mu\text{m}$ .

Figure 3. Immunohistochemical localization of ZBTB16 (A-D), SYCP3 (E-H), and GATA4 (I-L) in the seminiferous tubules at approximate stages of IV, VIII, and VII, respectively. A, E, I, immunostaining with the corresponding antibodies (red in A, E; green in I). B, F, J, staining with DAPI (blue). C, G, K, merged pictures. D, H, L, staining with HE after fluorescent staining. A-D, type A spermatogonia (A) were immunopositive, whereas intermediate spermatogonia (In) were

immunonegative. E-H, preleptotene spermatocytes (Pl) and pachyene spermatocytes (P) were immunopositive. I-L, Sertoli cells (Se) were immunopositive. Bars = 10  $\mu$ m.

Figure 4. Determination of stages (I-XII) and identification of cell types using the combination of IHC and PNA lectin histochemistry. Three- $\mu$ m thick sections were double-immunostained for GATA4 (green) and ZBTB16 (red), then stained with PNA-lectin histochemistry (green) and DAPI (blue). Se, Sertoli cells; A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M1, spermatocytes undergoing the first meiotic division; Sc, secondary spermatocytes; S1-16, step 1-16 spermatids. Bar = 10  $\mu$ m.

Figure 5. Determination of stages (I-XII) and identification of cell types using the combination of IHC and PNA lectin histochemistry. Three- $\mu$ m thick sections were double-immunostained for GATA4 (green) and SYCP3 (red), then stained with PNA-lectin histochemistry (green) and DAPI (blue). Se, Sertoli cells; A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M1, spermatocytes undergoing the first meiotic division; S1-16, step 1-16 spermatids. Bar = 10  $\mu$ m.

Figure 6. Schematic of acrosomal staining patterns with PNA lectin histochemistry and nuclear

immunostaining patterns for ZBTB16, SYCP3, and GATA4 at different stages (I-XII) of mouse spermatogenesis. PNA lectin is shown in green, GATA4 in light-green, ZBTB16 in red, and SYCP3 in pink. Dotted red and pink for ZBTB16 and SYCP3, respectively, indicate that immunoreactivity was weak and dot-like in distribution. Se, Sertoli cells; A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M, meiotic cells; S1-16, step 1-16 spermatids.

Figure 7. A comparison of seminiferous tubules in normal control (A-D), *nectin-2<sup>-/-</sup>* (E-H), and *nectin-3<sup>-/-</sup>* mice (I-O) at the representative stages shown in PAS-H staining (A-B, E-F, I-J), IHC for nectin-2 (red; C, G, K) and nectin-3 (red; D, H, L), and double IHC for SYCP3 (red) and GATA4 (green) in combination with PNA-lectin histochemistry (green) (M-O). Nuclei were stained with DAPI (blue; C-D, G-H, K-L, M-O). A-L: normal (A, E) and distorted (I) shapes of step 13 spermatids in stage I; abundant (B), fewer (F), and scarce (I) numbers of step 16 spermatids in stage VII; the presence (C, K) and absence (G) of nectin-2-immunoreactivity in Sertoli cells in stage I, as well as the presence (D, H) and absence (L) of nectin-3-immunoreactivity in step 13 spermatids in stage I were noted. M-O: stage VII (M), VIII (N), and IX (O) seminiferous tubules were distinguishable with the help of SYCP3 immunoreactivity in preleptotene (PI) spermatocytes at stage VIII, but not in those at stage VII, in leptotene (L) spermatocytes at stage IX, or in pachytene (P) spermatocytes at stages VII-IX, even in the absence of step 16 spermatids (S16) and with similar acrosomal patterns in step 7-9 spermatids (S7-9). Se, Sertoli cells immunopositive for

GATA4 (green). Bars = 20  $\mu\text{m}$  (A-L), 10  $\mu\text{m}$  (M-O).

Figure 8. An analysis of the numbers of different spermatogenic cell types in seminiferous tubules at different stages (I-XII) in normal control, nectin-2<sup>-/-</sup>, and nectin-3<sup>-/-</sup> mice. The numbers of all spermatogenic cell types in each stage were counted for a total of 322 (in control mouse), 197 (in nectin-2<sup>-/-</sup> mouse), or 220 (in nectin-3<sup>-/-</sup> mouse) seminiferous tubule sections obtained from a testis of a single representative mouse from the respective strains, and expressed as the mean cell number  $\pm$  SD per tubule in each stage. Data obtained in control mouse were the same as those in Table 1. A, type A spermatogonia; In, intermediated spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M, meiotic cells; S1-16, step 1-16 spermatids.

Figure 9. A comparison of the numbers of later step spermatids among normal control, nectin-2<sup>-/-</sup>, and nectin-3<sup>-/-</sup> mice. The mean numbers of step 10 and later spermatids in stage X-VIII seminiferous tubules were first calculated in every testis, as in Figure 8, and data obtained in 3 testes from 3 animals for each mouse strain were expressed as the average cell number  $\pm$  SD per tubule in each stage (n = 3). Differences in cell numbers between control, nectin-2<sup>-/-</sup>, and nectin-3<sup>-/-</sup> mice were analyzed with a one-way ANOVA followed by Bonferroni's posthoc test. \*, significantly lower than the numbers of corresponding spermatids in the same stages in control mice ( $P < 0.05$ , n = 3).



Table 1. The numbers of Sertoli cells and different spermatogenic cell types in each seminiferous tubule section belonging to a particular stage were counted for a total of 322 tubule sections in 2 tissue sections obtained from 2 separate portions of a testis from a single representative normal mouse, and expressed as the mean cell number  $\pm$  SD per tubule in each stage. No., the number of tubule sections belonging to each stage; A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia, Pl, preleptotene spermatocytes, L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; M, meiotic cells (cells undergoing the first and second meiotic divisions, including secondary spermatocytes); Average, the average cell number  $\pm$  SD per tubule of Sertoli cells, type A spermatogonia, intermediate spermatogonia, type B spermatogonia, Pl-Di spermatocytes, meiotic cells, step 1-12 spermatids, and step 13-16 spermatids in all tubule sections that contained the respective cell types. Within the groups of Sertoli cells, spermatogonia, spermatocytes, and spermatids, differences between the mean cell number of each cell type per tubule in different stages were analyzed with a one-way ANOVA followed by Scheffe's F test, and represented by the superscripts a, b, c, d, e, and f. A significant difference was observed between two values that had no common superscript ( $P < 0.01$ )

Table. 1 Numbers of different cell types in seminiferous tubules at different stages

Stage	No.	Sertoli	Spermatogonia			Spermatocytes			Spermatids	
			A	In	B	Pl-L-Z	P-Di	M	Step 1-12	Step 13-16
I	37	18.5 ± 3.8 <sup>a</sup>	7.9 ± 2.6 <sup>a</sup>				51.5 ± 7.7 <sup>a</sup>		153.0 ± 31.3 <sup>a</sup>	125.5 ± 23.2 <sup>bcde</sup>
II-III	50	18.2 ± 3.1 <sup>a</sup>	1.6 ± 1.4 <sup>b</sup>	10.8 ± 3.9 <sup>d</sup>			53.4 ± 8.5 <sup>a</sup>		149.8 ± 26.8 <sup>ab</sup>	110.3 ± 27.3 <sup>def</sup>
IV	30	17.9 ± 3.0 <sup>a</sup>	1.8 ± 1.7 <sup>b</sup>	13.4 ± 3.9 <sup>d</sup>			51.2 ± 8.4 <sup>a</sup>		147.6 ± 28.0 <sup>ab</sup>	97.6 ± 25.6 <sup>f</sup>
V	21	18.4 ± 3.5 <sup>a</sup>	2.5 ± 2.5 <sup>bc</sup>		24.6 ± 3.9 <sup>c</sup>		47.1 ± 9.0 <sup>a</sup>		148.7 ± 24.7 <sup>ab</sup>	98.8 ± 30.2 <sup>ef</sup>
VI	17	18.3 ± 3.2 <sup>a</sup>	2.4 ± 1.7 <sup>bc</sup>		28.1 ± 7.9 <sup>c</sup>		50.8 ± 7.8 <sup>a</sup>		146.8 ± 26.7 <sup>abc</sup>	110.8 ± 30.6 <sup>cef</sup>
VII	60	18.0 ± 4.0 <sup>a</sup>	2.4 ± 1.9 <sup>b</sup>			47.6 ± 7.8 <sup>a</sup>	51.2 ± 7.1 <sup>a</sup>		142.9 ± 28.2 <sup>ab</sup>	112.2 ± 24.7 <sup>def</sup>
VIII	28	17.9 ± 2.6 <sup>a</sup>	1.8 ± 1.5 <sup>b</sup>			49.7 ± 8.0 <sup>a</sup>	50.4 ± 9.9 <sup>a</sup>		145.5 ± 23.4 <sup>ab</sup>	95.4 ± 17.2 <sup>f</sup>
IX	29	18.3 ± 3.4 <sup>a</sup>	3.6 ± 2.4 <sup>bc</sup>			51.3 ± 7.9 <sup>a</sup>	55.7 ± 7.2 <sup>a</sup>		138.5 ± 35.5 <sup>abc</sup>	
X	14	17.4 ± 3.9 <sup>a</sup>	4.0 ± 1.9 <sup>bc</sup>			51.9 ± 8.4 <sup>a</sup>	57.5 ± 12.9 <sup>a</sup>		138.6 ± 43.1 <sup>abcd</sup>	
XI	10	18.6 ± 3.6 <sup>a</sup>	4.5 ± 1.9 <sup>abc</sup>			53.3 ± 6.2 <sup>a</sup>	61.3 ± 10.4 <sup>a</sup>		142.7 ± 20.4 <sup>abcd</sup>	
XII	26	18.1 ± 2.9 <sup>a</sup>	5.8 ± 2.6 <sup>ac</sup>			51.4 ± 7.1 <sup>a</sup>		96.3 ± 31.3 <sup>b</sup>	144.6 ± 30.4 <sup>abc</sup>	
Average		18.1 ± 3.4	3.3 ± 2.9	11.8 ± 4.1	26.1 ± 6.3		51.4 ± 8.7	96.3 ± 31.3	144.6 ± 29.8	108.8 ± 27.1

Figure 1

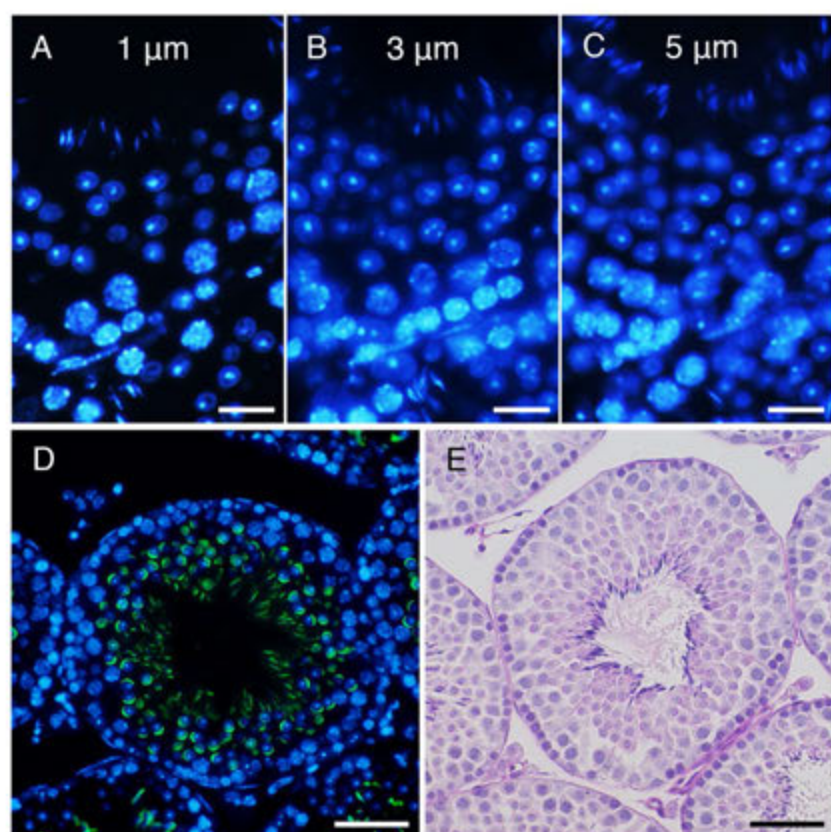




Figure 3

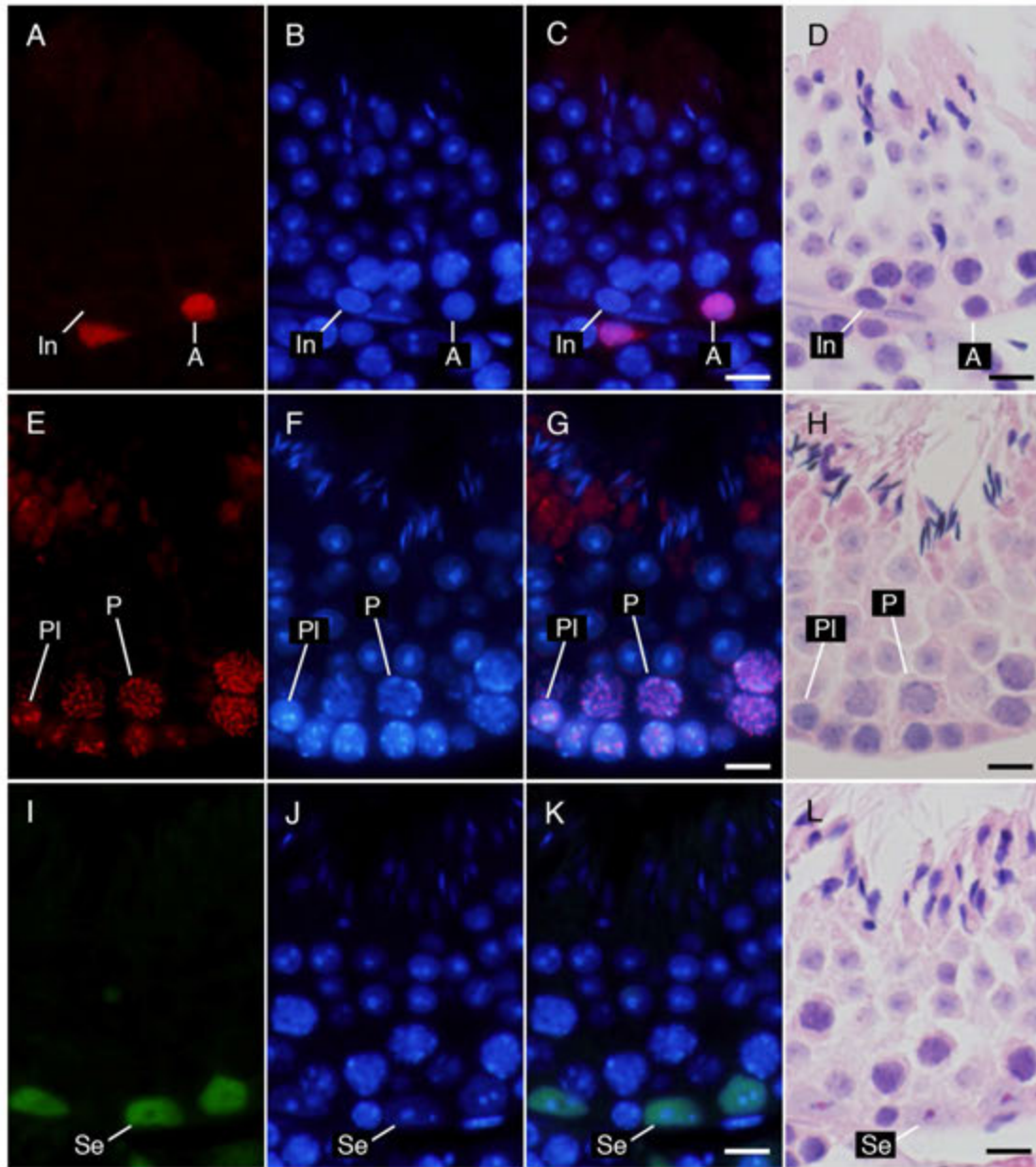




Figure 4

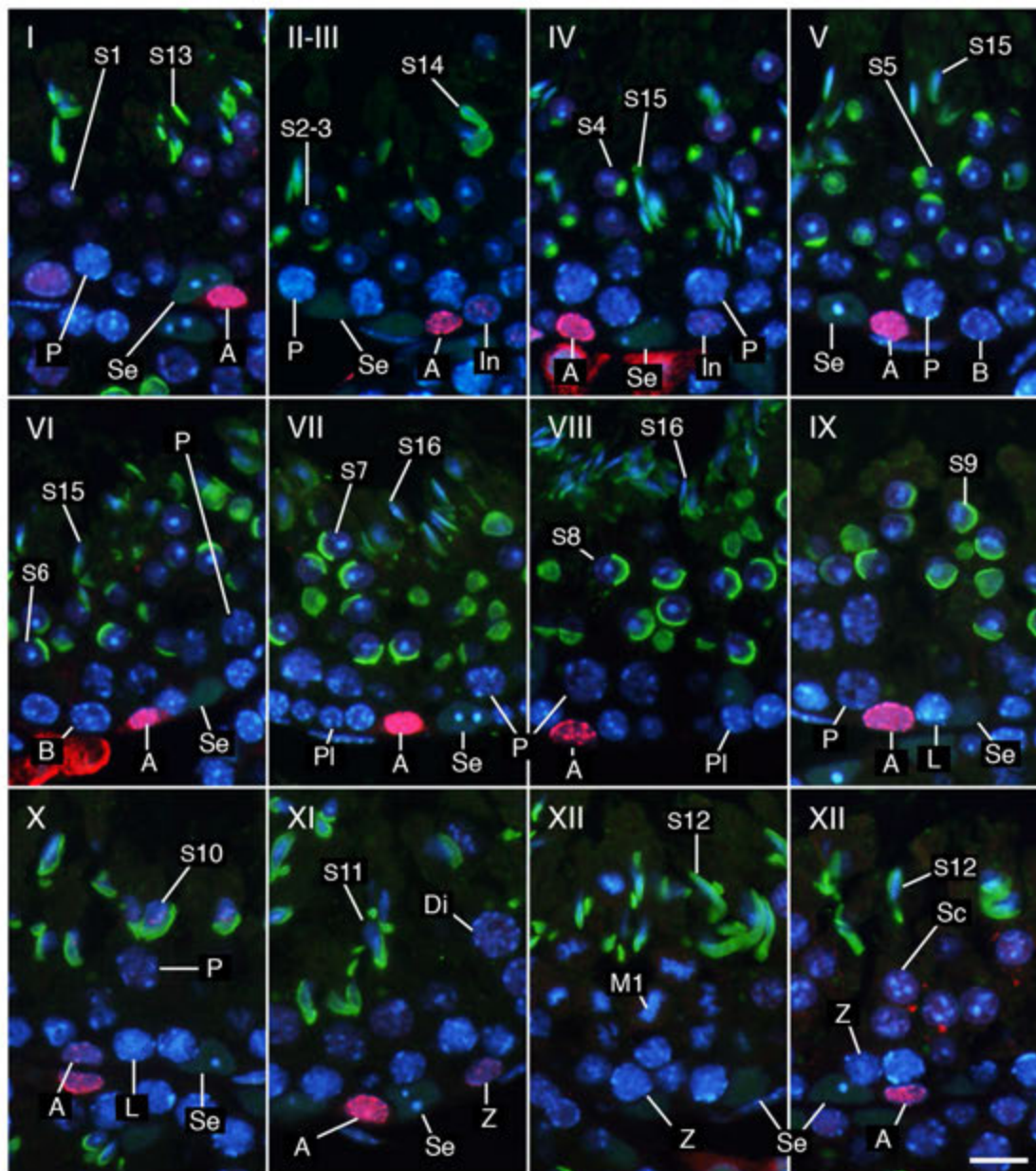


Figure 5

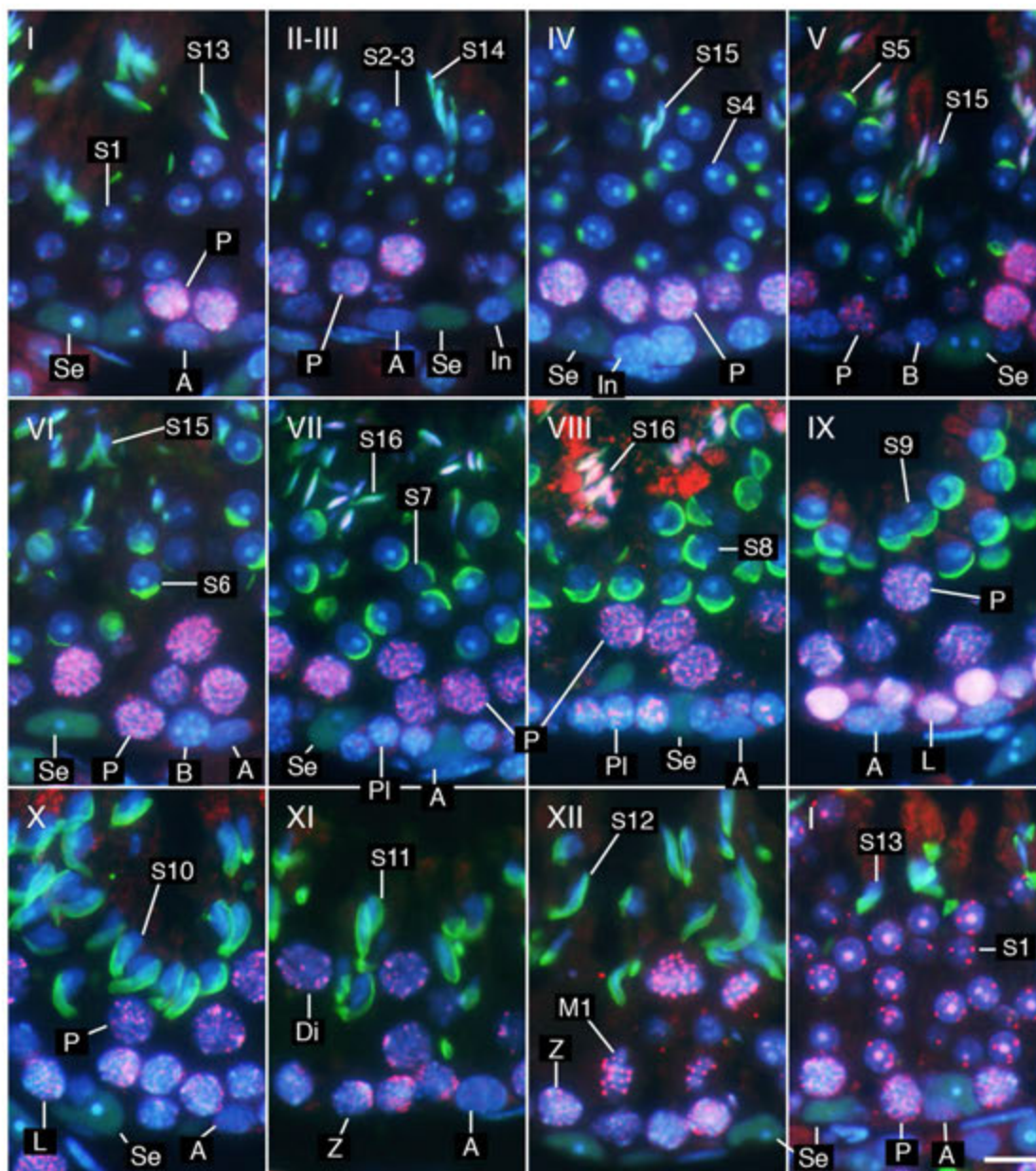


Figure 6

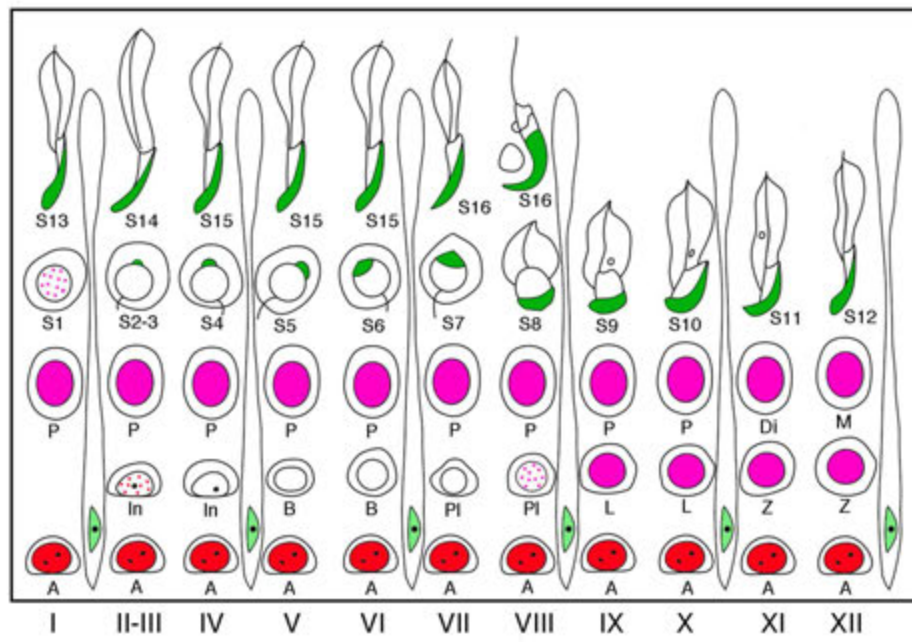




Figure 7

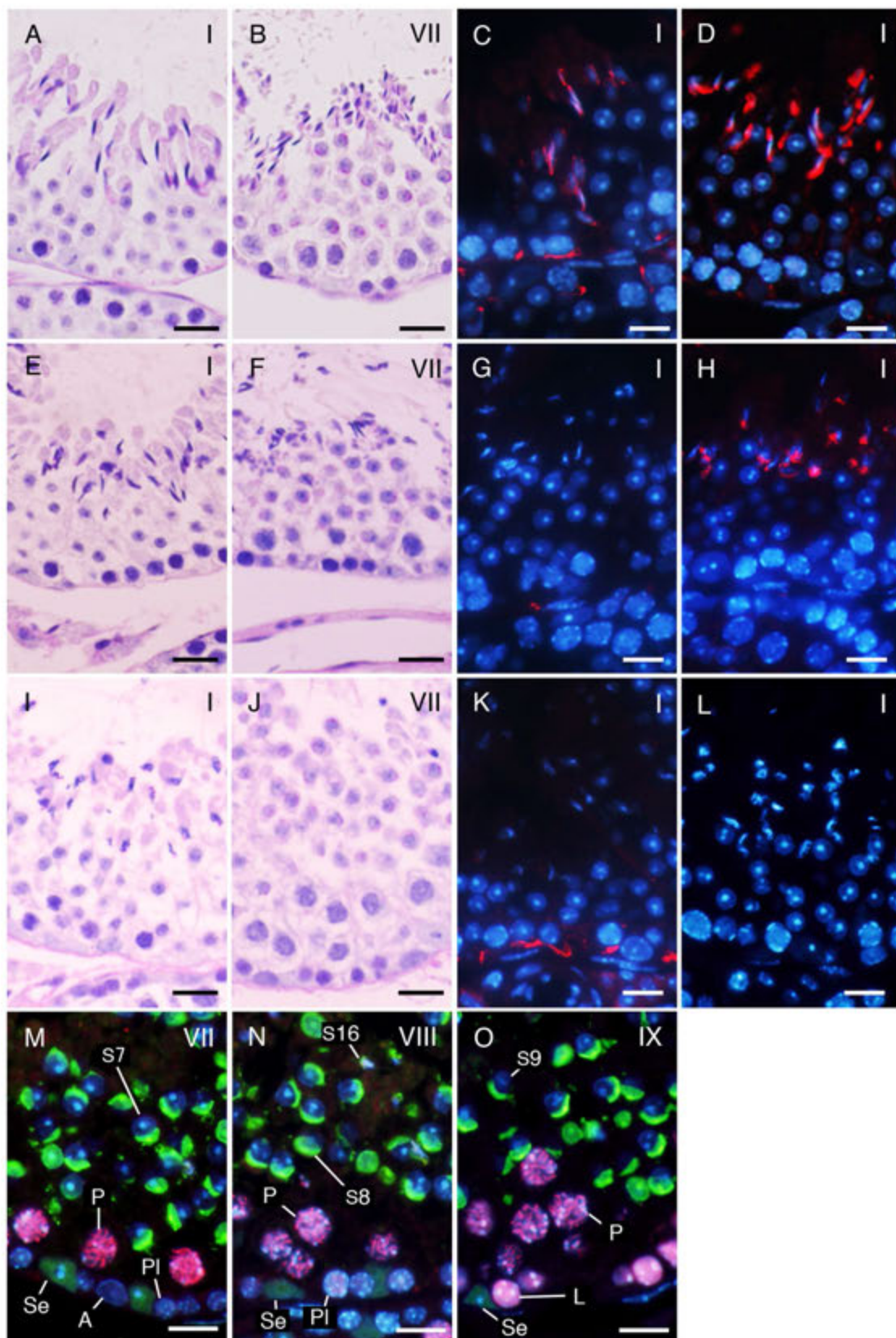


Figure 8

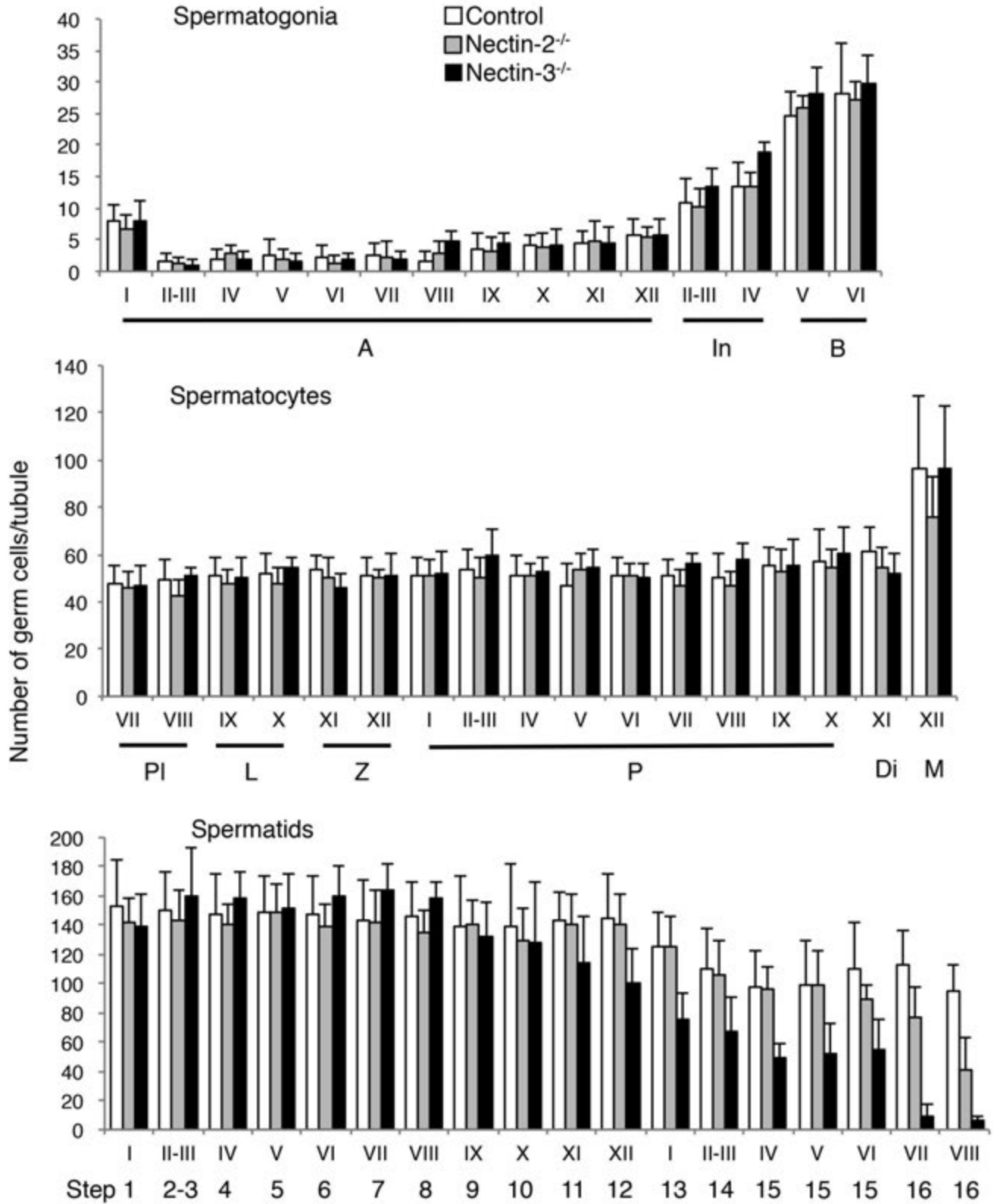


Figure 9

