—Original—

Seminiferous tubule development and germ cell proliferation occur asynchronously in the mouse

Fuyu Sato[1], Hiroya Nakamura[2] and Kazuei Matsubara[1, 2]*

[1] *Laboratory of Animal Sciences, Graduate School of Arts and Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan*
[2] *Laboratory of Animal Sciences, The United Graduate School of Agricultural Sciences, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan*

Abstract: Relatively few studies have examined the development of seminiferous tubules in the late embryonic and neonatal stages of mice, especially with regard to the proliferation of germ cells. In this study, the relationship between the development of seminiferous tubules and the proliferation of germ cells was investigated in mice from the late embryonic to neonatal period. Seminiferous tubule development and germ cell proliferation were evaluated histologically. The results show that from E15.5 to E16.5, the germ cell density decreased rapidly, but there was no change in the testis cord area. From E16.5 to E17.5, although the testis cord area reduced by half, the germ cell density was maintained even though the germ cell number did not change in this period. These results indicate that the testis cord greatly elongates from E15.5 to P0, especially in the period from E15.5 to E17.5. They also indicate that germ cell proliferation is not synchronous with seminiferous tubule expansion and that elongation of the testis cord occurs from the late embryonic stage until birth. Using these observations, a model of germ cell and seminiferous tubule development was constructed.

Key words: Germ cells, Spermatogenesis, Seminiferous tubules, Testis, Gonocytes

Introduction

The testis is composed of a large number of tubules in which germ-line cells divide and differentiate to produce sperm. Seminiferous tubules contain both germ cells and supporting cells such as Sertoli cells. The tubules form a closed system that is separated from the stromal cells by a basement membrane, where spermatogenesis takes place. Spermatogenesis is the process by which undifferentiated spermatogonia, located in the basement membrane of the seminiferous tubules, self-renew and differentiate then move toward the lumen to become mature sperm.

Sperm are produced through multiple stages of proliferation, meiosis, and maturation. Spermatogenesis continues throughout life and can be classified into the first wave of initial spermatogenesis from gonadal establishment to puberty, and the adult steady-state that follows the establishment of self-renewing spermatogonial stem cells.

Primordial germ cells (PGCs) first appear in the extra-embryonic mesoderm on embryonic day 7.0 (E7.0) in mice, and proliferate and migrate through the hindgut to the genital ridge (GR) [1]. PGCs enter the GR and they are in cell-cycle arrest at G0/G1 between E13.5 and E16.5 [2, 3]. After birth, germ cell mitosis restarts [2]. Mitotically-arrested germ cells (gonocytes) migrate from the center of the testis cord via pseudopodia from E18.5 to just after birth [4] and attach to the basement membrane [5]. This attachment is important for gonocyte survival and differentiation [6, 7].

Seminiferous tubules in the fetal stage are called testis cords. Testis cord formation in mouse embryonic gonads begins with activation of the *Sry* gene (sex-determining region of the Y chromosome) [8] in Sertoli progenitor cells [9, 10]. The expression of *Sry* is transient and has immunohistologically been shown to occur in the mid-region of the male gonad at E11. Its expression extends to the anterior and posterior edges of the gonad by E11.5, then disappears from the mid-region to the anterior and

Received: October 7, 2021
Accepted: January 8, 2022
*To whom correspondence should be addressed.
e-mail: kazuei@iwate-u.ac.jp

posterior edges by E12.0 [11–13]. Immediately after the expression of *Sry* (E11.5), SOX9 (Sry-related HMG box 9), a downstream factor of *Sry* and a Sertoli cell differentiation factor, is activated [14, 15]. SOX9 not only forms a cell-autonomous feedback loop that maintains its own expression, but also acts on downstream factors that act to increase its expression [16, 17]. SOX9 promotes the proliferation and differentiation of Sertoli cells and contributes to the formation of the testis cord.

Cell migration from the mesonephros to the gonad is essential for testis cord formation. Mesonephric cells, mainly endothelial cells [18, 19], migrate to the gonad between E11.5~16.5 and differentiate into myoblasts and vascular endothelial cells [20–22]. Removal of the mesonephros or installation of a physical barrier between the mesonephros and gonad to inhibit cell migration results in the absence of testis cord structures [21, 23–25]. Several mouse models have indicated a relationship between defects of vasculogenesis and abnormal testis cord formation. It has been proposed that the formation of a vascular network by endothelial cells is required for testis cord formation [26–28].

Structural analyses of the mouse seminiferous tubule have identified other important events in testis cord formation. Combes *et al.* [29] reported that the testis cords observed at E12.5 ran perpendicular to the long axis of the gonads. The number of testis cords does not change at E12.5~14.5, but their mean diameter doubles from E12.5 to E13.5. In response to the development of cord structure, the testis cord elongates and expands within the gonad, undergoing morphological changes such as bending and fusion of the cords. Nakata *et al.* [30, 31] reported that the seminiferous tubules of adult mice run around the circumference of the testis in a highly tortuous manner and have a regular layered structure, starting with those leading to the upper end of the testicular network. Similar to the fetal stage, this high degree of bending and fusion allows the seminiferous tubules to elongate. Postnatally, the seminiferous tubules maintain the basic structures that were established in the fetal period. However, our understanding of the structural development of seminiferous tubules from the late embryonic to neonatal periods is comparatively poor. In addition, the relationship between germ cell proliferation and differentiation and seminiferous tubule development is unclear. Elucidation of this relationship would provide a basis for future morphological studies of the testis, and provide new insights into testis cord development in the late embryonic stages. Increased insights into tubule development and germ cell differentiation should be of benefit to the field of reproductive medicine, such as in the evaluation of spermatogenesis and the creation of infertility models.

In this study, mouse testes from the embryonic to postnatal stages were analyzed histologically. A model of the development of seminiferous tubules and germ cells was constructed using our observations of changes in the seminiferous tubule structure, germ cell density, and distance between germ cells.

Materials and Methods

Animals

Pregnant ICR mice were euthanized by cervical dislocation and their uteri were dissected and the fetuses collected. Male neonatal ICR pups less than 7 days old were anesthetized on ice and decapitated. Gonads were collected from the male ICR mice aged E12.5 to P6 using dissecting needles.

Observations and immunohistology

The gonads were fixed overnight at 4°C in 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd.), washed with PBS, dehydrated with ethanol, and embedded in paraffin blocks. For histological examination, sections (3 μm) were made for each sample and stained with hematoxylin and eosin (H&E). For immunofluorescence staining, sections (4 μm) were rehydrated after deparaffinization, and washed twice for 5 minutes with PBS containing 0.1% Tween 20 (PBS-T) (Wako Pure Chemical Industries, Ltd.). Permeabilization was performed by immersing the sections in cooled methanol for 10 minutes, and then washing again with PBS-T for 5 minutes. The sections were then immersed in Blocking One (Nacalai Tesque) for 1 hour at room temperature. Next, rabbit anti-DDX4 (DEAD-Box Helicase 4)/MVH (Mouse Vasa Homologue) polyclonal antibody (1:1000, Abcam: Cat. No. ab13840), rabbit anti-SOX9 polyclonal antibody (1:500, Sigma: Cat. No. HPA001758) and rat anti-Ki67 monoclonal antibody (1:500, Invitrogen: Cat.No.14-5698-82) were added as the primary antibodies and the sections were incubated overnight at 4°C. After washing three times with PBS-T for 5 minutes, Alexa594-conjugated donkey anti-rabbit IgG (1:500, Abcam: Cat. No. ab150064) and Alexa488-conjugated donkey anti-rat IgG (1:500, Abcam: Cat. No. ab150153) were applied and the sections counterstained with DAPI (1:1000, Wako Pure Chemical Industries, Ltd.: Cat. No. 340-07971); secondary antibody staining was performed at 37°C for 1 hour. Finally, the sections were washed three times with PBS-T for 5 minutes, and 90% glycerol/PBS was applied. The sections were observed under a fluorescence microscope

(Olympus). DDX4/MVH was used as a germ cell specific marker. This marker is expressed in the fetus from late migration stage PGCs to gonocytes, and in the adult from spermatogonia to spermatids. SOX9 and Ki67 were used as Sertoli cell and proliferation markers, respectively.

Germ cell and Sertoli cell density

In each section, ten circular seminiferous tubules were randomly selected from males aged E14.5 to P6 (n ≥ 3). The number of DDX4/MVH-positive cells and SOX9-positive cells were counted in each selected tubule. Cross-sectional areas of the seminiferous tubules were measured using Image J (Ver. 1.52q) image analysis software, and the germ cell density and Sertoli cell density of each seminiferous tubule cross-section was calculated.

Proliferative activity of Sertoli cells

The number of SOX9-positive cells was counted in each section from ages E14.5 to P2 (n ≥ 5). Subsequently, the number of SOX9+/Ki67+ cells was counted and the proliferation index of Sertoli cells was calculated.

Distances between germ cells

Testes from mice aged E14.5 to P6 were sectioned parallel to the long axis of the seminiferous tubules. Neighboring germ cells were randomly selected from seminiferous tubules running longitudinally through the sections. The distance between the DDX4/MVH maximal expression area of each germ cell was measured using Image J (Ver. 1.52q) (intercellular distances; n = 150).

The concentration of seminiferous tubules

HE stained sections of E14.5 to P4 testes were captured at the same magnification, and the number of seminiferous tubules in each image was counted (n ≥ 4).

Statistical analysis

The germ cell density, Sertoli cell density, seminiferous tubule cross-sectional area, distance between germ cells at each age, proliferation index of Sertoli cells and number of seminiferous tubules are shown as means ± standard deviation (SD). Welch's t-test was used to compare the different groups.

Results

Germ cell settlement and localization in the gonads

Testis cords were identified in the GR at the age of E12.5 and many MVH/DDX4-positive germ cells were present (Fig. 1A, i). As embryonic age progressed, the number of testis cords increased and the number of MVH/DDX4-positive cells observed per testis cord decreased (Fig. 1A, ii and iii). From late embryonic age to just after birth, germ cells located in the center of the testis cord began to migrate to the basal membrane and become attached (Fig. 1A, iv and v). The number of MVH/DDX4-positive cells attached to the basement membrane increased after birth. (Fig. 1A, vi)

Seminiferous tubule development and germ cell density

In tubules from mice aged E14.5 to P6, the concentration of DDX4/MVH-positive cells and the areas of the seminiferous tubules were measured. The density of germ cells in each testis cord significantly decreased from E15.5 to E16.5 ($P < 0.01$) then remained constant until P0 (Fig. 1B). The density of germ cells decreased significantly again at the age of P2 ($P < 0.01$) and then increased until P6. At the age of E17.5, the area of the testis cord declined significantly ($P < 0.01$) and thereafter the area was almost constant until P2 (Fig. 1C). After P2, the area increased significantly ($P < 0.01$~0.05).

Change of inter-cell distances

To determine how the changes in germ cell density were produced, thin sections were cut parallel to the long axis of seminiferous tubules (Fig. 2A). Germ cells were found to be densely packed in the period immediately after the formation of the testis cord until birth (Fig. 2A, i–iii). In the postnatal stage, the distance between germ cells seemed to increase (Fig. 2A, iv). Then, mitosis restarted and germ cells increased, and the distance between germ cells gradually decreased (Fig. 2A, v and vi). The distance between germ cells was almost constant from E14.5 to P0 (Fig. 2B). However, the distance increased significantly ($P < 0.01$), nearly 2-fold, in the neonatal stage from P0 to P2. After P2, the distance reduced significantly ($P < 0.01$~0.05) to the same level as that seen in the embryonic stage.

Proliferative activity of Sertoli cells and the complexity of the seminiferous tubule structure

To evaluate whether the decrease in germ cell density during the fetal stage was due to the elongation of the testis cord, the proliferative activity of Sertoli cells was examined (Fig. 3A). The number of Ki67-positive Sertoli cells was small in the E14.5 testis (Fig. 3A, i), but subsequently, a large number of these cells were observed (Fig. 3A, ii–v). The density of Sertoli cells increased significantly ($P < 0.01$) from E14.5 to E18.5 (Fig. 3B). After E18.5 the density reduced significantly ($P < 0.01$). The proliferation index of Sertoli cells increased sig-

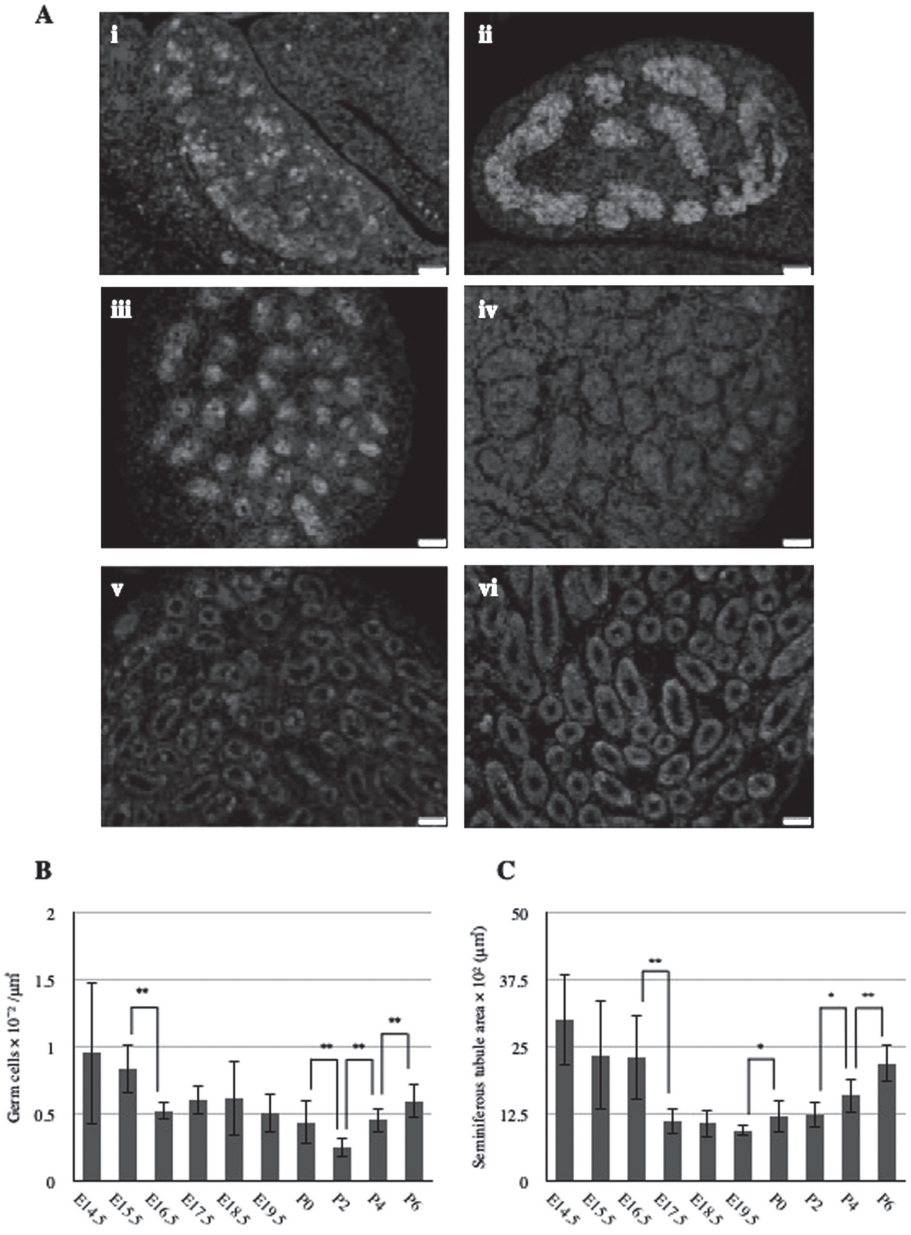

Fig. 1. Structural changes in the gonads and germ cell density.
A, immunofluorescence was performed using testes sectioned vertical to the long axis of the seminiferous tubules from mice aged E12.5 to P6. DAPI (blue) and anti MVH/DDX4 antibody (red) are shown in all images. (i) E12.5; (ii) E14.5; (iii) E17.5; (iv) P0; (v) P2; (vi) P6. Scale bars are 50 μm. B, changes in germ cell density at different stages. **P* < 0.05, ***P* < 0.01. Bar shows mean ± SD (n = 10). C, change in seminiferous tubule area at different stages. **P* < 0.05, ***P* < 0.01. Bar shows mean ± SD (n = 10).

nificantly (*P* < 0.01~0.05) from E14.5 to E18.5 (Fig. 3C). The proliferation index also decreased significantly (*P* < 0.01~0.05) after E18.5. The change in testis cord numbers and the complexity of the cord structure were assessed by counting the number of cross-sectional testis cords (Fig. 4). The concentration of cross-sectional testis cords seemed to increase as the fetal age progressed (Fig. 4A). Moreover, the number of cross-sectional testis cords in the same magnification view increased significantly from E14.5 to P0 (*P* < 0.05) (Fig. 4B).

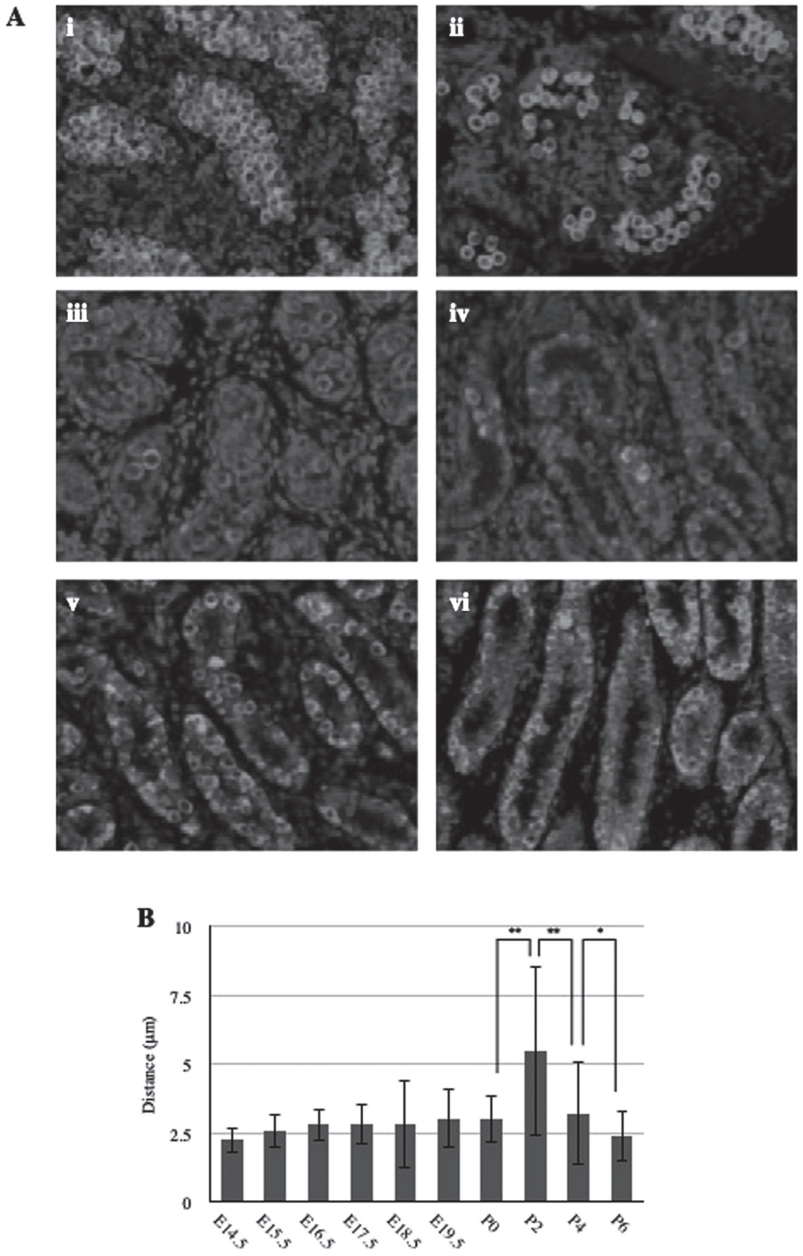

Fig. 2. Distance between germ cells.
A, immunofluorescence was performed on testis sections sectioned parallel to the long axis of the seminiferous tubules from mice aged E14.5 to P6. DAPI (blue) and anti MVH/DDX4 antibody (red) are shown in all images. (i) E14.5; (ii) E18.5; (iii) P0; (iv) P2; (v) P4; (vi) P6. B, average distance between germ cells at different developmental stages. *$P < 0.05$, **$P < 0.01$. Bar shows mean ± SD (n = 150).

Discussion

In this study, a histological investigation of the relationship between seminiferous tubule development and germ cell proliferation in mice was performed. At the embryonic age of E14.5, germ cells were densely packed within the testis cord. However, later in development, the concentration of germ cells in the testis cord decreased. From E15.5 to E16.5, the germ cell density decreased rapidly, but there was no change in the testis cord area. From E16.5 to E17.5, although the testis cord area reduced by one half, germ cell density was maintained.

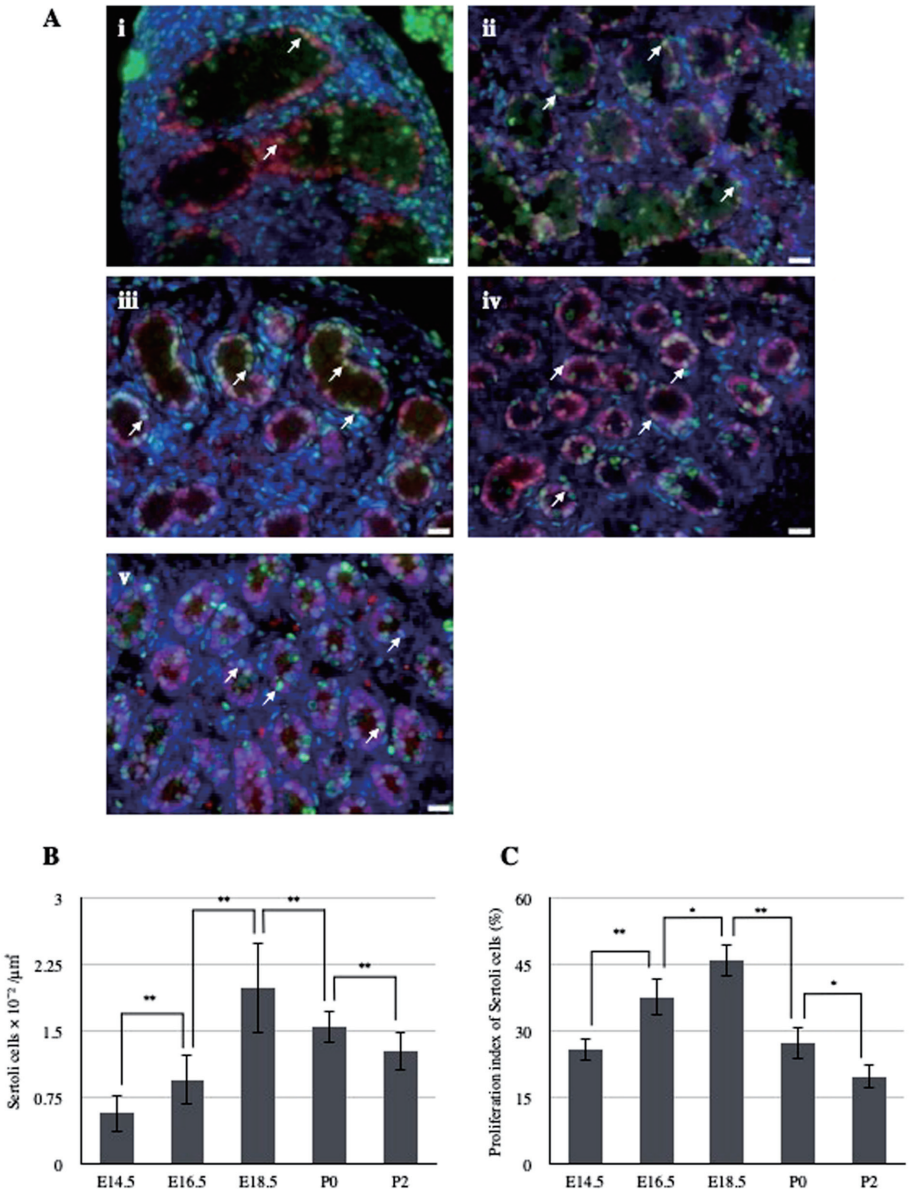

Fig. 3. Proliferative activity of Sertoli cells.
A, immunofluorescence was performed using testis sections from mice aged E14.5 to P2. DAPI (blue), anti SOX9 antibody (red) and anti Ki67 antibody (green) are shown in all images. The white arrow indicates a Sertoli cell during active phases of the cell cycle (SOX9+/Ki67+). (i) E14.5; (ii) E16.5; (iii) E18.5; (iv) P0; (v) P2. Scale bars are 20 μm. B, changes in Sertoli cell density at different stages. *$P < 0.05$, **$P < 0.01$. Bar shows mean ± SD (n = 10). C, proliferation index of Sertoli cells. *$P < 0.05$, **$P < 0.01$. Bar shows mean ± SD (n = 10).

Gonocytes enter mitotic arrest during E14.5 to E16.5 and remain in this state until after birth. Moreover, the apoptosis rate of gonocytes in mitotic arrest is quite low or zero [32]. This means the gonocyte number does not change during this time. Therefore, these results suggest that testis cord enlargement and/or increase in the cord number occur during the period from E15.5 to E17.5. The timing of the expansion of the testis cords observed in this study is consistent with previous reports, and it is generally believed that the proliferation of Sertoli cells is involved in the elongation and expansion of the testis cord. Archambeault and Yao [33] suggested that Leydig

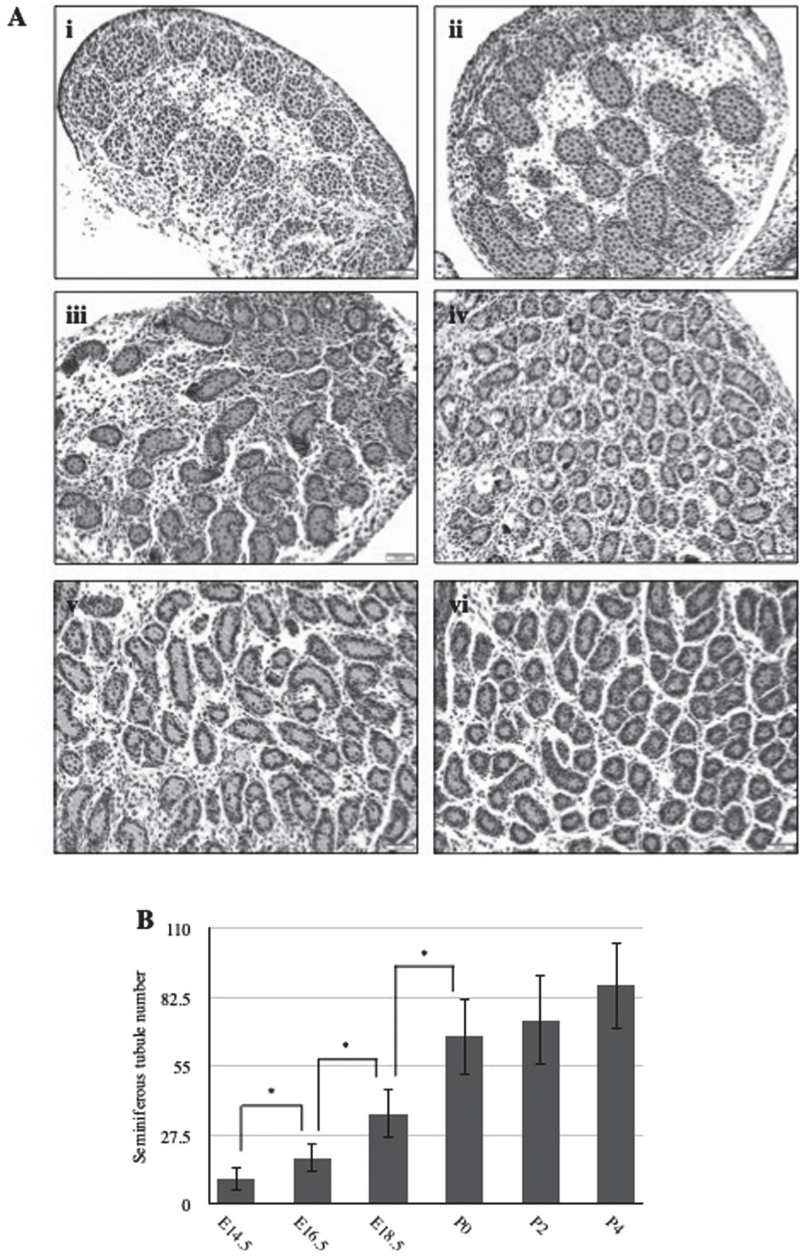

Fig. 4. Cross-sections of seminiferous tubules and change in the number of seminiferous tubules.
A, representative images of sections stained with hematoxylin and eosin from E14.5 to P4 testes. (i) E14.5; (ii) E16.5; (iii) E18.5; (iv) P0; (v) P2; (vi) P4. Scale bars are 50 μm. B, change in the number of seminiferous tubule cross-sections. **P* < 0.05. Bar shows mean ± SD (n ≥ 5).

cells are involved in the elongation and expansion of the testis cord after E15.5 via modification of the proliferation rate of Sertoli cells. Sertoli cells also have an FSH receptor [34], and binding to FSH produced after E17.5 promotes the proliferation of Sertoli cells [35]. Furthermore, insulin-like growth factor 1 (IGF1) enhances the cell proliferation pathway through FSH signaling [36]. Collectively, these reports suggest that the proliferative activity of Sertoli cells increases in the late embryonic stage before birth, and several reports have confirmed the rate of proliferation of Sertoli cells peaks in the period from E16.5 to just after birth, and declines slowly thereafter [2, 37, 38]. In rats, a variety of factors such as TGFα, GDNF and Interleukin-1 have been shown to be involved

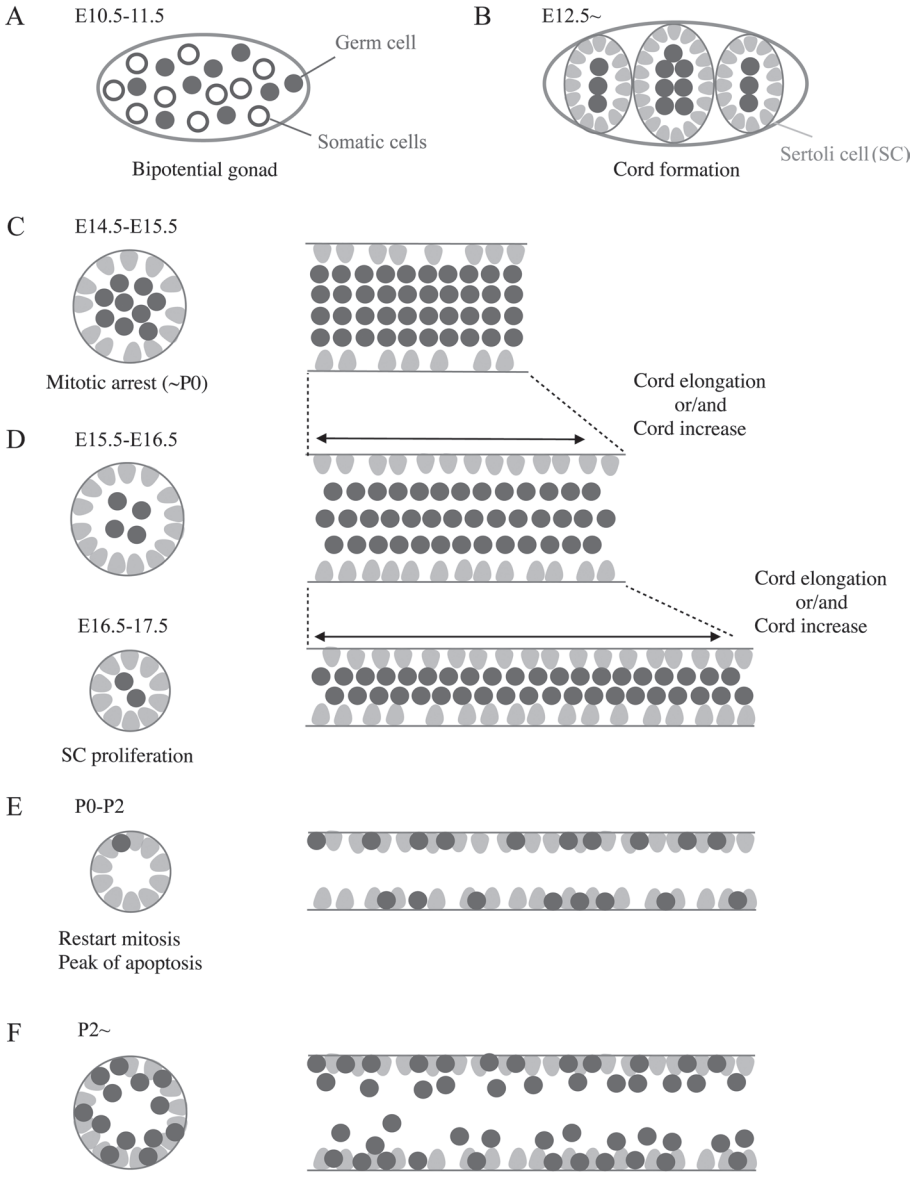

Fig. 5. Model for germ cell proliferation during the development of seminiferous tubules.
A: Germ cells are mixed with gonadal somatic cells within the genital ridge.
B: Sertoli cells are generated and surround the germ cells to form a testis cord. In this period, vasculogenesis and migration of endothelial cells occur, forming the basic structure of the testis.
C: Mitosis-arrested germ cells are densely present in the testis cord.
D: Cord elongation and/or cord increase occur due to Sertoli cell proliferation.
E: After birth, germ cells decrease due to an increase in apoptosis, even though mitosis resumes. Germ cells migrate from the lumen of seminiferous tubules and attach to the basement membrane.
F: Germ cells increase due to an increase in mitosis.

in Sertoli cell proliferation [39–48]. Investigating the expression of these factors in the late embryonic stages of mice may lead to a better understanding of the regulatory system behind the proliferation of Sertoli cells.

The seminiferous tubule area and germ cell density did not change much between the embryonic ages of E17.5 and E19.5. This suggests they do not significantly change during the development of the testis cord. The distance between germ cells was measured to confirm whether the testis cord extends during the fetal stage; however,

the distance was found to be constant until birth. The Sertoli cell density, the proliferative activity of Sertoli cells and the density of testis cords were all evaluated in this study. The density of Sertoli cells increased from E14.5 to E18.5 and the proliferation index also increased. Thereafter, the density and the index decreased. In addition, the number of cross-sectional cords increased significantly during the period from E14.5 to P0. Thus, an increase in the number of testis cords and/or complication of the testis cord structure such as branching and bending due to elongation would occur in this time. These results suggest that the development of seminiferous tubules may be less rapid than before E17.5, even though testis cords elongate during the period from E15.5 to P0. Moreover, testis cord development couldn't be assessed by measuring the distance between germ cells.

Though the area of seminiferous tubules did not change, the density of germ cells decreased from P0 to P2. This change in density is consistent with a previous study [49]. The distance between germ cells increased significantly, and this increase in distance can be explained by an increase in the apoptosis index. Mitosis restarts after birth, and at the same time the apoptosis index increases. The apoptosis index reaches a peak on P2, and thereafter it decreases reaching a score as low as adult mice on P3 [50]. Thus, the germ cell number decreased due to apoptosis and the distance between germ cells became greater. After P4, the density increased significantly, and the distance decreased. This change would be due to an increase in mitosis as the mitosis index increased. However, it was not clear in this study whether or not the expansion of the tubule area was due to an increase in mitosis. Thus, further experiments are needed to determine whether germ cell proliferation or differentiation influences the expansion of the seminiferous tubules.

In conclusion, the results indicate that the testis cords greatly elongate from E15.5 to P0, especially in the period from E15.5 to E17.5. This result strongly supports previous reports that the testis cords greatly elongate during fetal life. Furthermore, the data indicates that the germ cell proliferation and seminiferous tubule enlargement occur asynchronously during fetal life. The results suggest a model for seminiferous tubule development (Fig. 5). This model describes in detail the growth of the testis cords and germ cell proliferation by age and provides a basis for future research into testicular development. Elucidating the details of normal testis cord development will further understanding of normal and pathological testicular morphologies and spermatogenesis.

Disclosures

Conflict of interest: The authors declare no conflict of interest.

Human and Animal rights: This study did not involve any studies with human subjects performed by the any of the authors.

Animal studies: All institutional and national guidelines for the care and use of laboratory animals were followed.

Approval by ethics committee: This study was conducted with the approval of the Iwate University Animal Experiment Committee (A201626).

References

1) Gomperts, M., Wylie, C. and Heasman, J. (1994): Primordial germ cell migration. Ciba Found. Symp., 182, 121–134; discussion 134–139.
2) Nagano, R., Tabata, S., Nakanishi, Y., Ohsako, S., Kurohmaru, M. and Hayashi, Y. (2000): Reproliferation and relocation of mouse male germ cells (gonocytes) during prespermatogenesis. Anat. Rec., 258, 210–220.
3) Western, P.S., Miles, D.C., van den Bergen, J.A., Burton, M. and Sinclair, AH. (2008): Dynamic regulation of mitotic arrest in fetal male germ cells. Stem Cells, 26, 339–347.
4) McGuinness, M.P. and Orth, J.M. (1992): Reinitiation of gonocyte mitosis and movement of gonocytes to the basement membrane in testes of newborn rats in vivo and in vitro. Anat. Rec., 233, 527–537.
5) Clermont, Y. and Perey, B. (1957): Quantitative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat., 100, 241–267.
6) Hasthorpe, S., Barbic, S., Farmer, P.J. and Hutson, J.M. (1999): Neonatal mouse gonocyte proliferation assayed by an *in vitro* clonogenic method. J. Reprod. Fertil., 116, 335–344.
7) McGuinness, M.P. and Orth, J.M. (1992): Gonocytes of male rats resume migratory activity postnatally. Eur. J. Cell. Biol., 59, 196–210.
8) Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N. Goodfellow, P. and Lovell-Badge, R. (1990): A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature, 346, 245–250.
9) Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R. and Goodfellow, P.N. (1990): A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature, 346, 240–244.
10) Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. and Lovell-Badge, R. (1991): Male development of chromosomally female mice transgenic for Sry. Nature, 351, 117–121.
11) Albrecht, K.H. and Eicher, E.M. (2001): Evidence that Sry is

expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev. Biol., 240, 92–107.
12) Bullejos, M. and Koopman, P. (2001): Spatially dynamic expression of Sry in mouse genital ridges. Dev. Dyn., 221, 201–205.
13) Kidokoro, T., Matoba, S., Hiramatsu, R., Fujisawa, M., Kanai-Azuma, M., Taya, C., Kurohmaru, M., Kawakami, H., Hayashi, Y., Kanai, Y. and Yonekawa, H. (2005): Influence on spatiotemporal patterns of a male-specific Sox9 activation by ectopic Sry expression during early phases of testis differentiation in mice. Dev. Biol., 278, 511–525.
14) Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H. and Koopman, P. (1996): A male-specific role for SOX9 in vertebrate sex determination. Development., 122, 2813–2822.
15) Sekido, R. and Lovell-Badge, R. (2008): Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature, 453, 930–934.
16) Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., Chaboissier, M.C., Poulat, F., Behringer, R.R., Lovell-Badge, R. and Capel, B. (2006): Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. PLoS Biol., 4, e187.
17) Moniot, B., Declosmeni, l.F., Barrionuevo, F., Scherer, G., Aritake, K., Malki, S., Marzi, L., Cohen-Solal, A., Georg, I., Klattig, J., Englert, C., Kim,Y., Capel, B., Eguchi, N., Urade, Y., Boizet-Bonhoure, B. and Poulat, F. (2009): The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. Development, 136, 1813–1821.
18) Cool, J., Carmona, F.D., Szucsik, J.C. and Capel, B. (2008): Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad. Sex. Dev., 2, 128–133.
19) Combes, A.N., Wilhelm, D., Davidson, T., Dejana, E., Harley, V., Sinclair, A. and Koopman, P. (2009): Endothelial cell migration directs testis cord formation. Dev. Biol., 326, 112–120.
20) Wartenberg, H. (1978): Human testicular development and the role of the mesonephros in the origin of a dual Sertoli cell system. Andrologia, 10, 1–21.
21) Buehr, M., Gu, S. and McLaren, A. (1993): Mesonephric contribution to testis differentiation in the fetal mouse. Development, 117, 273–281.
22) Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R. and Capel, B. (1997): Male-specific cell migration into the developing gonad. Curr. Biol., 7, 958–968.
23) McLaren, A. and Buehr, M. (1990): Development of mouse germ cells in cultures of fetal gonads. Cell Differ. Dev., 31, 185–195.
24) Merchant-Larios, H., Moreno-Mendoza, N. and Buehr, M. (1993): The role of the mesonephros in cell differentiation and morphogenesis of the mouse fetal testis. Int. J. Dev. Biol., 37, 407–415.
25) Tilmann, C. and Capel, B. (1999): Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. Development, 126, 2883–2890.
26) Albrecht, K.H., Capel, B., Washburn, L.L. and Eicher, E.M. (2000): Defective mesonephric cell migration is associated with abnormal testis cord development in C57BL/6J XY (Mus domesticus) mice. Dev. Biol., 225, 26–36.
27) Brennan, J., Tilmann, C. and Capel, B. (2003): Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. Genes. Dev., 17, 800–810.
28) Yao, H.H., Aardema, J. and Holthusen, K. (2006): Sexually dimorphic regulation of inhibin beta B in establishing gonadal vasculature in mice. Biol. Reprod., 74, 978–983.
29) Combes, A.N., Lesieur, E., Harley, V.R., Sinclai,r A.H., Little, M.H., Wilhelm, D. and Koopman, P. (2009): Three-dimensional visualization of testis cord morphogenesis, a novel tubulogenic mechanism in development. Dev. Dyn., 238, 1033–1041.
30) Nakata, H., Wakayama, T., Sonomura, T., Honma, S., Hatta, T. and Iseki, S. (2015): Three-dimensional structure of seminiferous tubules in the adult mouse. J. Anat., 227, 686–694.
31) Nakata, H., Sonomura, T. and Iseki, S. (2017); Three-dimensional analysis of seminiferous tubules and spermatogenic waves in mice. Reproduction, 154, 569–579.
32) Wang, R.A., Nakane, P.K. and Koji, T. (1998): Autonomous cell death of mouse male germ cells during fetal and postnatal period. Biol. Reprod., 58, 1250–1256.
33) Archambeault, D.R. and Yao, H.H. (2010): Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. Proc. Natl. Acad. Sci. U S A, 107, 10526–10531.
34) Heckert, L. and Griswold, M.D. (1993): Expression of the FSH receptor in the testis. Recent. Prog. Horm. Res., 48, 61–77.
35) Japon, M.A., Rubinstein, M. and Low, MJ. (1994): In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J. Histochem. Cytochem., 42, 1117–1125.
36) Pitetti, J.L., Calvel, P., Zimmermann, C., Conne, B., Papaioannou, M.D., Aubry, F., Cederroth, C.R., Urner, F., Fumel, B., Crausaz, M., Docquier, M., Herrera, P.L., Pralong, F., Germond, M., Guillou, F., Jégou, B. and Nef, S. (2013): An essential role for insulin and IGF1 receptors in regulating sertoli cell proliferation, testis size, and FSH action in mice. Mol. Endocrinol., 27, 814–827.
37) Vergouwen, R.P., Jacobs, S.G., Huiskamp, R., Davids, J.A. and De Rooij, D.G. (1991): Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J. Reprod. Fertil., 93, 233–243.
38) Baker, P.J. and O'Shaughnessy, P.J. (2001): Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice. Reproduction, 122, 227–234.
39) Slegtenhorst-Eegdeman, K.E., de Rooij, D.G., Verhoef-Post, M., van de Kant, H.J., Bakker, C.E., Oostra, B.A., Grootegoed, J.A. and Themmen, A.P. (1998): Macroorchidism in FMR1 knockout mice is caused by increased Sertoli cell proliferation during testicular development. Endocrinology, 139, 156–162.
40) Hu, J., Shima, H. and Nakagawa, H. (1999): Glial cell line-derived neurotropic factor stimulates sertoli cell proliferation in the early postnatal period of rat testis development.

Endocrinology, 140, 3416–3421.
41) Levine, E., Cupp, A.S., Miyashiro, L. and Skinner, M.K. (2000): Role of transforming growth factor-alpha and the epidermal growth factor receptor in embryonic rat testis development. Biol. Reprod., 62, 477–490.
42) Petersen, C., Frysa, B., Boitani, C. and Sder, O. (2000): Transforming growth factor-alpha stimulates Sertoli cell proliferation in vitro. Andrologia, 32, 62–63.
43) Petersen, C., Boitani, C., Frysa, B. and Sder, O. (2001): Transforming growth factor-alpha stimulates proliferation of rat Sertoli cells. Mol. Cell. Endocrinol., 181, 221–227.
44) Petersen, C., Boitani, C., Frysa, B. and Sder, O. (2002): Interleukin-1 is a potent growth factor for immature rat sertoli cells. Mol. Cell. Endocrinol., 186, 37–47.
45) Petersen, C., Frysa, B. and Sder, O. (2004): Endotoxin and proinflammatory cytokines modulate Sertoli cell proliferation in vitro. J. Reprod. Immunol., 61, 13–30.
46) Petersen, C., Svechnikov, K., Frysa, B. and Sder, O. (2005): The p38 MAPK pathway mediates interleukin-1-induced Sertoli cell proliferation. Cytokine., 32, 51–59.
47) Cardoso, L.C., Nascimento, A.R., Royer, C., Porto, C.S. and Lazari, M.F. (2010): Locally produced relaxin may affect testis and vas deferens function in rats. Reproduction, 139, 185–196.
48) Nascimento, A.R., Pimenta, M.T., Lucas, T.F., Royer, C., Porto, C.S. and Lazari, M.F. (2012): Intracellular signaling pathways involved in the relaxin-induced proliferation of rat Sertoli cells. Eur. J. Pharmacol., 691, 283–291.
49) Li, R., Vannitambly, A., Zhang, J.G., Fehmel, E.L., Southwell, B.R. and Hutson, J.M. (2015): Oct4-GFP expression during transformation of gonocytes into spermatogonail stem cells in the perinatal mouse testis. J. Pediatr. Surg., 50, 2084–2089.
50) Drumond, A.L., Meistrich, M.L. and Chiarini-Garcia, H. (2011): Spermatogonial morphology and kinetics during testis development in mice: a high-resolution light microscopy approach. Reproduction, 142, 145–155.