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Gene expression ontogeny of spermatogenesis in the marmoset uncovers primate characteristics during testicular development

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ABSTRACT

Mammalian spermatogenesis has been investigated extensively in rodents and a strictly controlled developmental process has been defined at cellular and molecular levels. In comparison, primate spermatogenesis has been far less well characterized. However, important differences between primate and rodent spermatogenesis are emerging so it is not always accurate to extrapolate findings in rodents to primate systems. Here, we performed an extensive immunofluorescence study of spermatogenesis in neonatal, juvenile, and adult testes in the common marmoset (*Callithrix jacchus*) to determine primate-specific patterns of gene expression that underpin primate germ cell development. Initially we characterized adult spermatogonia into two main classes; mitotically active C-KIT$^+$Ki67$^+$ cells and mitotically quiescent SALL4$^+$PLZF$^+$LIN28$^+$DPPA4$^+$ cells. We then explored the expression of a set of markers, including PIWIL1/MARWI, VASA, DAZL, CLGN, RanBPM, SYCP1 and HAPRIN, during germ cell differentiation from early spermatocytes through round and elongating spermatids, and a clear program of gene expression changes was determined as development proceeded. We then examined the juvenile marmoset testis. Markers of gonocytes demonstrated two populations; one that migrates to the basal membrane where they form the SALL4$^+$ or C-KIT$^+$ spermatogonia, and another that remains in the lumen of the seminiferous tubule. This later population, historically identified as pre-spermatogonia, expressed meiotic and apoptotic markers and were eliminated because they appear to have failed to correctly migrate. Our findings provide the first platform of gene expression dynamics in adult and developing germ cells of the common marmoset. Although we have characterized a limited number of genes, these results will facilitate primate spermatogenesis research and understanding of human reproduction.

Introduction

Mammalian spermatogenesis is a strictly organized developmental process involved in the passage of genetic material. Spermatogenesis originates from a population of spermatogonia called spermatogonial stem cells that undergo the sequential processes of mitotic and meiotic spermiogenesis to produce spermatozoa. In rodents, many aspects of spermatogenesis have been analyzed including cell morphology, organization, and gene expression (Hermo et al., 2010). These studies have led to the identification of numerous molecular signatures to discriminate each stage of spermatogenesis. By contrast, owing to less accessibility to tissue samples and ethical and legal issues, there are fewer such comparative studies on primate spermatogenesis.

An increasing number of studies have revealed that primates and rodents have a different developmental program for spermatogenesis (YDBIO6681Hermann et al., 2010; McKinnell et al., 2013; Schlatt and Ehmcke, 2014). For example, the stem cell system underlying spermatogenesis differs between primates and rodents. In humans there are two distinct types of undifferentiated spermatogonia; quiescent A_{dark} spermatogonia, which are

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believed to serve as reserve stem cells, and renewing A_{pale} spermatogonia, which proliferate regularly (YDBIO|6681Clermont, 1963, 1966, 1969, 1972). This categorization is also the case in New-World (YDBIO|6681Eildermann et al., 2012; Millar et al., 2000) and Old-World monkeys (YDBIO|6681Ehmcke et al., 2006; Hermann et al., 2010), but is not incorporated into rodent spermatogenesis that does not enclose reserve stem cells (Schlatt and Ehmcke, 2014).

Differences in the molecular profile are also evident between primate and rodent spermatogenesis. In our previous study, we compared the molecular signatures of spermatogenic cells in mouse and common marmoset (*Callithrix jacchus*) with regard to DNA methylation status, transcripts, and protein expression (Lin et al., 2012). We revealed that in marmosets the *NANOG* and *OCT4* promoters exhibit DNA hypomethylation and hypermethylation, respectively, unlike mouse homologs, which display an opposite DNA methylation pattern (YDBIO|6681Imamura et al., 2006; Western et al., 2010). Additionally, we found that marmoset spermatogonia express DAZL but not VASA protein, while both proteins are expressed in mouse spermatogonia. These observations reveal that simple extrapolation of rodent insights to primates is not appropriate. Hence, investigation of primates should be considered. However, very little is known about the molecular dynamics of primate spermatogenesis. There is, therefore, a fundamental need for a suitable non-human primate model.

As a model animal to investigate primate spermatogenesis, we have focused on a New-World monkey, the common marmoset that is native to the Atlantic coastal forests in northeastern Brazil. Among primates this species has attracted much attention in recent years as it is an excellent experimental animal for biomedical research. It has a small body size (20–30 cm height; 350–400 g weight in adulthood), fast sexual maturation (around 1.5 years old), breeds throughout the year with a prolificacy of 40–80 offspring for a female life, and is physiologically similar to humans (YDBIO|6681Kishi et al., 2014; Mansfield, 2003; Okano et al., 2012). In addition, our group has succeeded in producing offspring with assisted reproduction technologies (YDBIO|6681Takahashi et al., 2014, Tomioka et al., 2012), and creating a transgenic marmoset with germline transmission (Sasaki et al., 2009), expanding their potential for genetic research. Furthermore, compared with other non-human primates, marmosets and humans share a similar organization of spermatogenesis and testicular development (YDBIO|6681Millar et al., 2000; Mitchell et al., 2008; Saunders et al., 1996; Wistuba et al., 2003) as both species retain gonocytes in the postnatal testes (YDBIO|6681Albert et al., 2010; Sharpe et al., 2003). To date, some studies have examined gene expression of marmoset germ cells but only focused on either a single or a few genes (e.g. OCT4, SALL4, and VASA) at a particular developmental stage (YDBIO|6681Eildermann et al., 2012; Lin et al., 2012; McKinnell et al., 2013). A comprehensive gene expression atlas of marmoset spermatogenesis remains to be defined.

In this study, to develop the atlas of gene expression dynamics in the course of marmoset spermatogenesis, we dissected a sequence of gene expression associated with spermatogenic processes. Extensive immunofluorescent microscopy of neonatal, juvenile, and adult testes elucidated not only gene expression benchmarks but also a unique developmental event occurring in the juvenile period. We believe that our findings could serve as the first molecular platform to facilitate primate spermatogenesis research, and be a first step for unveiling primate-specific developmental programs.

Materials and methods

Animals and tissue collection

All the common marmosets used in this study were housed in cages with dimensions of $82 \times 61 \times 160\ cm^3$. This study was approved by Institutional Animal Care and Use Committee of the Central for Experimental Animals (CIEA) and was performed in accordance with CIEA guidelines that agree with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan (2006).

RT-PCR

Total RNA prepared with TRIzol reagent was isolated using the RNeasy Mini kit (Qiagen, 74106) according to the manufacturer's instructions. First-strand cDNA was synthesized with SuperScript-tIII reverse transcriptase (Invitrogen, 18080-044) and oligo-dT_{18} primer. The primers were designed based on sequences in the marmoset genome database (http://genome.wustl.edu/tools/blast/index) as described in Supplementary Table S1. Subsequent PCR analysis was performed with Ex Taq Hot Start Version (TaKaRa, RR006A). All experiments were performed semiquantitatively at three different escalation cycles, and only representative images are shown in the result.

Western blot

Marmoset tissues were homogenized in lysis buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA (pH 8.0) and 1% Nonidet-P40 (NP-40). After centrifugation, the supernatants were diluted with sample buffer and boiled. Ten microgram of total proteins per lean was separated on 7.5% SDS-PAGE and transferred nitrocellulose (Whatman, 10 401 196) or PVDF (GE Healthcare, RPN303F) membranes. The membranes were blocked at room temperature with 5% (w/v) skim milk for 30 min. After five washes with 0.1% PBS-T, the membranes were incubated with primary antibodies at room temperature for 1 h or overnight at 4 °C. The following primary antibodies were used: culture supernatant of anti-MARWI hybridoma cells (1:1, 2D9) (Hirano et al., 2014), anti-GST (1:1000, Santa Cruz Biotechnology, sc-138), anti-FLAG (M2) (1:5000, Sigma, F3165), anti-GAPDH (14C10) (1:3000, Cell Signaling, 2118) antibodies. HRP-conjugated anti-mouse IgG (1:5000, MP BIOMEDICALS, 55558) or anti-rabbit IgG (1:5000, Immuno-Biological Laboratories, 17502) were used as secondary antibodies. Blots were developed using ECL detection reagents (Amersham Biosciences, RPN2109).

Immuno-electron microscopy

Frozen sections from adult marmoset testes were incubated with anti-MARWI antibody (1:200, 2D9-1H6) for 4 days at 4 °C followed by incubation with nanogold conjugated anti-mouse secondary antibody (1:100, Invitrogen, A-24921) for 1 day at 4 °C. After fixation in 2.5% glutaraldehyde for 10 min and enhancement with HQ-Silver enhancement kit (Nanoprobes Inc., 2012) for 12 min at 25 °C in a dark room, sections were post-fixed with 0.5% osmium tetroxide, dehydrated through ethanol, acetone, and QY1, and embedded in Epon. Ultrathin sections (70 nm thickness) were prepared and stained with 2% uranyl acetate and 80 mM lead citrate (Reynolds methods) for 15 and 10 min respectively. The sections were observed under a transmission electron microscope (JEOL model 1230), while images were taken with a Digital Micrograph 3.3 (Gatan Inc.).

Immunofluorescence microscopy of marmoset testis

Testes were isolated from neonatal (1-day-old), juvenile (10-month-old), and adult marmosets (2, 3, 4, 5, 9, 10-year-old). Each testis was fixed in 4% (w/v) paraformaldehyde, dehydrated, embedded in paraffin wax and sectioned at 5 μm thickness. The sections were de-waxed with xylene, rehydrated in a graded alcohol series, and washed with distilled water. For immunofluorescence analyses, the sections were subjected to antigen retrieval by autoclave at 105 °C for at least 5 min in 1 × Target Retrieval Solution (Dako, S1699). The sections were blocked at room temperature with 5% (w/v) skim milk for 30 min and incubated with the primary antibody at 4 °C overnight in a humidified chamber. The following primary antibodies were used: rabbit anti-SALL4 (1:100, Abcam, ab29112), mouse anti-SALL4 (1:100, Abcam, ab57577), rabbit anti-Ki67 (1:100, Leica Biosystems, NCL-Ki67p), mouse anti-Ki67 (1:100, Leica Biosystems, NCL-L-Ki67-MM1), rabbit anti-C-KIT (1:100, Abcam, ab32363), mouse anti-PLZF (1:100, Abcam, ab104854), goat anti-LIN28A (1:100, R&D systems, AF3757), rabbit anti-DPPA4 (1:50, Abcam, ab154642), mouse anti-MARWI (1:50, 2D9-1H6), rabbit anti-DDX4 (1:50, Abcam, ab13840), mouse anti-DDX4 (1:50, Abcam, ab27591), rabbit anti-DAZL (1:100, Abcam, ab34139), rabbit anti-Calmegin (1:100, Abcam, ab171971), rabbit anti-RanBPM (1:100, Abcam, ab78127), rabbit anti-SYCP1 (1:100, Abcam, ab15087), goat anti-RNF98 (1:100, Abcam, ab4836), mouse anti-phospho-histone H2A.X (ser139) (1:100, Millipore, 05-636), rabbit anti-active Caspase 3 (1:100, Abcam, ab13847), and rabbit anti-PARP1(Asp214) (1:100, Cell Signaling, 9541S) antibodies. Secondary antibodies included Alexa Fluor 488 goat anti-mouse IgG (1:500; Invitrogen, A-11029), Alexa Flour 488 donkey anti-mouse IgG (1:500, Invitrogen, A-21202), Alexa Flour 488 donkey anti-goat IgG (1:500, Invitrogen, A-11055), Alexa Fluor 555 goat anti-rabbit IgG (1:500, Invitrogen, A-21429), Alexa Flour 555 donkey anti-rabbit IgG (1:500, Invitrogen, A-31572), and Alexa Flour 555 donkey anti-goat IgG (1:500, Invitrogen, A-21432). Nuclei were stained with 10 ng/ml Hoechst 33342. For each experiment, a negative control was included in which the primary antibody had been omitted. Images were captured using a Zeiss confocal microscope system (LSM700).

Results

Molecular subtyping of spermatogonia in adult marmoset testis

The common marmoset has two subtypes of undifferentiated A spermatogonia; one is mitotically quiescent A_{dark} spermatogonia, and the other is mitotically active A_{pale} spermatogonia (YDBIO|6681Hermann et al., 2010; Millar et al., 2000). These two types of spermatogonia have been classically recognized by their characteristic nuclear staining patterns and morphological criteria, but their molecular signatures remain unclear. To discriminate marmoset spermatogonia subtypes by gene expression, we carried out immunofluorescence analyses of adult marmoset testes, referring to known spermatogonia markers in other mammalian species. First, we focused on the Ki67 antigen, an indicator for dividing cells (Gerdes et al., 1984), because the major cytological difference between A_{dark} and A_{pale} spermatogonia is their mitotic activity (YDBIO|6681Dym et al., 2009; Ehmcke and Schlatt, 2006). The distribution of Ki67$^+$ cells in the seminiferous tubules was compared with cells expressing pluripotency transcription factor SALL4, which is known to be a type A spermatogonium marker (Eildermann et al., 2012). Although both SALL4 and Ki67 were detected in marmoset spermatogonia, the SALL4$^+$ and Ki67$^+$ spermatogonia clearly constituted different populations (Fig. 1A and Supplementary Fig. S1).

We next examined the expression of C-KIT protein, which is also known to mark spermatogonia in marmosets (von Schonfeldt et al., 1999) and rhesus macaques (Hermann et al., 2009). C-KIT protein was mostly expressed in the Ki67$^+$ spermatogonia, albeit a part of C-KIT$^+$ spermatogonia did not display Ki67 staining (Fig. 1B and Supplementary Fig. S2). By contrast, the expression of SALL4 and C-KIT proteins seemed to be mutually exclusive (Fig. 1C) while other spermatogonial markers, PLZF (YDBIO|6681Hermann et al., 2007; Hermann et al., 2009) and LIN28 (Aeckerle et al., 2012) proteins, were basically co-expressed with SALL4 protein (Fig. 1D and E), which is similar to the case of mouse homologs (YDBIO|6681Gassei and Orwig, 2013; Hobbs et al., 2012). DPPA4 (Maldonado-Saldivia et al., 2007) protein was also specified in the SALL4$^+$ spermatogonia (Fig. 1F). Taken together, it seems that marmoset spermatogonia can be classified into two major subtypes, which are SALL4$^+$PLZF$^+$LIN28$^+$DPPA4$^+$ mitotically quiescent cells, and C-KIT$^+$Ki67$^+$ mitotically active cells, possibly suggesting A_{dark} and A_{pale} spermatogonia respectively.

Marmoset PIWIL1, a component of chromatoid body

Next, we were interested in identifying gene expression of the spermatocytes and spermatids in adult marmoset testis. We have already found that germ cell-specific RNA helicase VASA is expressed from primary spermatocytes to round spermatids (Lin et al., 2012); hence, we intended to examine the expression of other marker genes. We particularly focused on the PIWI-family proteins, which associate with PIWI-interacting RNAs (piRNAs) to protect genomic integrity from transposable elements and, therefore, are expected to be conserved across mammalian species (Siomi et al., 2011). RT-PCR analysis of marmoset tissues identified the expression of all four PIWI genes (*PIWIL1*, *2*, *3*, and *4*) in testis together with the related genes (Supplementary Fig. S3), as reported in humans (Sasaki et al., 2003). Among them, PIWIL1 was subjected to detailed investigation because mouse PIWIL1/MIWI is expressed from pachytene spermatocytes to the elongating spermatids (Deng and Lin, 2002) and interacts with VASA protein (Kuramochi-Miyagawa et al., 2004).

To examine the expression of marmoset homologue of PIWIL1, which we designated as MARWI, we utilized an anti-MARWI monoclonal antibody derived from a hybridoma line 2D9 (Supplementary Fig. S4) (Hirano et al., 2014). In mice, PIWIL1/MIWI protein is concentrated in the chromatoid body, a germ cell-specific cloud-shaped granule, together with VASA protein in the cytoplasm of pachytene spermatocytes and spermatids (Kotaja and Sassone-Corsi, 2007). We carried out immunofluorescence analysis of marmoset testis and found that the subcellular localization of MARWI protein corresponded to that of VASA protein (Fig. 2A), in accordance with our recent report (Hirano et al., 2014). Notably, large granular staining of MARWI and VASA proteins was clearly observed in the round spermatids. The localization of MARWI protein was further examined by immuno-electron microscopy that identified marmoset chromatoid bodies with high electron density, in which MARWI protein was incorporated (Fig. 2B).

Molecular staging of marmoset spermatogenesis from spermatocytes to spermatids

To characterize developmental stage-specific gene expression in marmoset spermatocytes and spermatids, we examined MARWI protein in combination with other germ cell-related genes. As reported previously (YDBIO|6681Lin et al., 2012; Ruggiu et al., 2000), an RNA-binding protein DAZL was expressed in marmoset spermatogonia and spermatocytes (Fig. 3A). By contrast, MARWI protein appeared at the pachytene spermatocyte stage and the expression continued throughout the round and elongating

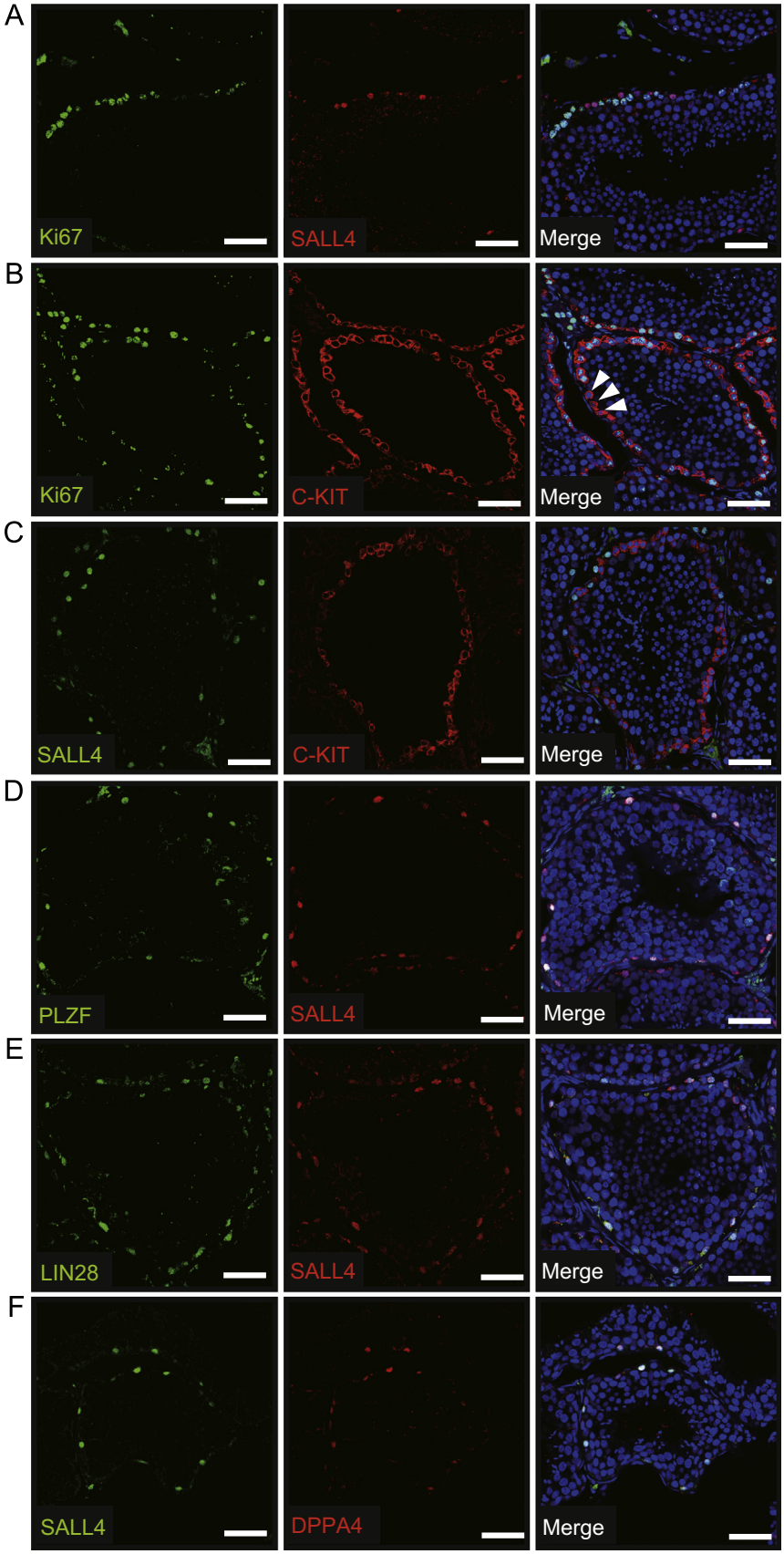

Fig. 1. Spermatogonial subtypes in the adult marmoset testis. Confocal images of adult marmoset testis sections co-stained with antibodies against spermatogonial markers, including (A) anti-Ki67 and anti-SALL4 antibodies, (B) anti-Ki67 and anti-C-KIT antibodies, (C) anti-SALL4 and anti-C-KIT antibodies, (D) anti-PLZF and anti-SALL4 antibodies, (E) anti-LIN28 and anti-SALL4 antibodies, and (F) anti-SALL4 and anti-DPPA4 antibodies. In the image of (B), arrowheads indicate C-KIT^{+}Ki67^{-} spermatogonia. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

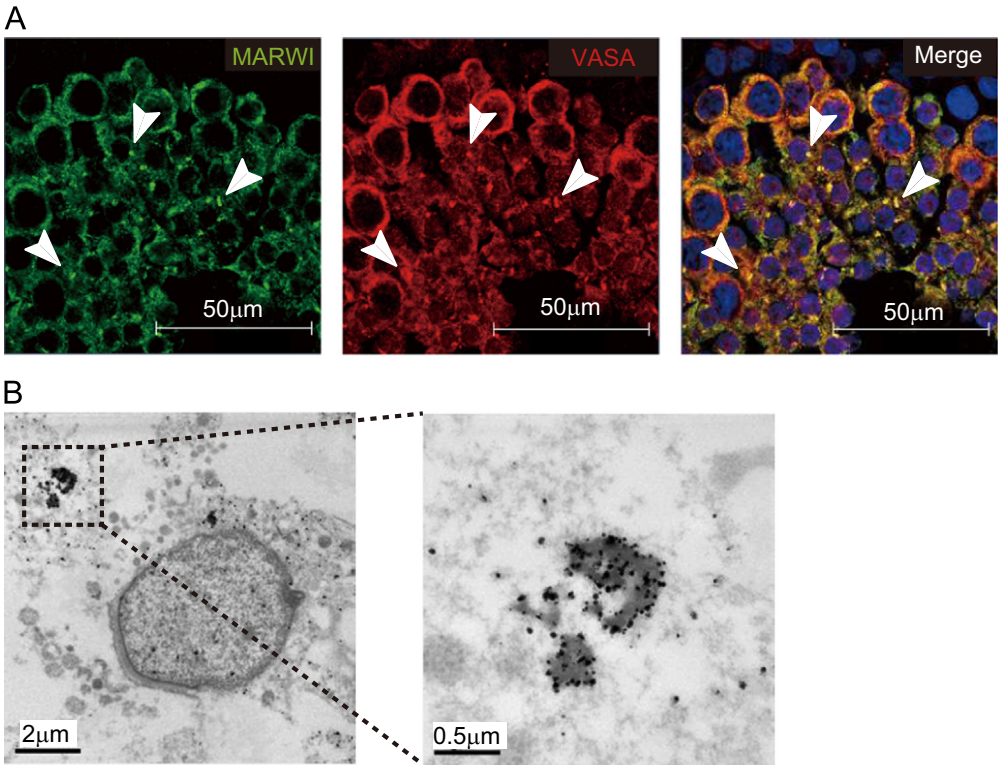

Fig. 2. Subcellular localization of marmoset PIWIL1 protein in chromatoid body. (A) Confocal images of adult testis frozen sections co-stained with anti-MARWI and anti-VASA antibodies. Arrowheads indicate chromatoid body-like cytoplasmic granules consisted of condensed MARWI and VASA proteins. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm. (B) Immuno-electron microscopic analysis of adult testis frozen section stained with anti-MARWI antibody. MARWI proteins were detected in chromatoid bodies with high electron density in the cytoplasm.

spermatid stages; therefore only the pachytene spermatocytes expressed both proteins. During mouse spermatogenesis, a testis-specific chaperone protein Calmegin (CLGN) is expressed in the pachytene spermatocytes to the elongating spermatids, and disappears in the mature spermatids (Yoshinaga et al., 1999). Marmoset CLGN protein first appeared in the early spermatocytes modestly, and then culminated at the pachytene spermatocyte stage in accordance with the onset of MARWI expression (Fig. 3B). The expression was also observed in the round spermatids but not in the elongating spermatids.

The expression of VASA and MARWI proteins mostly overlapped during the course of marmoset spermatogenesis as both proteins were expressed in the pachytene spermatocytes to the round spermatids (Fig. 3C). However, in the elongating spermatids, MARWI, but not VASA, was detected. RanBPM, a scaffold protein that is known to interact with VASA protein (YDBIO|6681Puverel et al., 2011; Shibata et al., 2004), also exhibited the expression pattern similar to VASA in adult marmoset testis. The nucleocytoplasmic expression of marmoset RanBPM was observed in MARWI$^+$ spermatocytes and round spermatids but not in the elongating spermatids (Fig. 3D). On the other hand, a synaptonemal complex protein SYCP1 and a haploid germ cell-specific RING finger protein HAPRIN (YDBIO|6681Kitamura et al., 2005; Kitamura et al., 2003) displayed more restricted expression. Like cynomolgus monkeys and humans (YDBIO|6681Pousette et al., 1997; Yamauchi et al., 2009), marmoset SYCP1 was preferentially expressed in pachytene spermatocytes (Fig. 3E). HAPRIN was detected in the acrosomal region of spermatids following the initiation of spermiogenesis (Fig. 3F).

Meiosis-associated nuclear structures of marmoset germ cells during spermatogenesis

Meiotic male germ cells can be characterized by their unique chromosome structures, such as the XY body (de Vries et al., 2012) and synaptonemal complex assembly (Page and Hawley, 2004), at meiosis prophase I. The former is a chromatin domain composed of condensed sex chromosomes while the latter is involved in pairing homologous chromosomes. These structures can be marked by phosphorylated histone γH2AX and SYCP1 proteins respectively, and are organized predominantly in the pachytene spermatocytes of mice (YDBIO|6681Mahadevaiah et al., 2001; Monesi, 1965) and humans (YDBIO|6681de Vries et al., 2012; Pousette et al., 1997). Similarly, in marmoset testis, the dot-shaped single staining of γH2AX was detectable in the nuclei of DAZL$^+$VASA$^+$ pachytene spermatocytes while it was widely diffuse in the nuclei at earlier stages (Fig. 4A and B). The cells with a pronounced γH2AX focus also expressed SYCP1 protein (Fig. 4C and D). Thus, during marmoset spermatogenesis the nuclear localization of γH2AX and SYCP1 proteins clearly identified the formation of the XY body and synaptonemal complex in pachytene spermatocytes, but not in the early spermatocytes or the round spermatids.

Juvenile marmoset testis contains the unique germ cells expressing meiosis-associated genes

One of the unique features of primate reproduction is the existence of long pre-pubertal and pubertal periods before reaching sexual maturity (YDBIO|6681Chemes, 2001; Kelnar et al., 2002; Rey et al., 1993). At the juvenile phase in marmosets, we found a unique group of germ cells located in the luminal region of seminiferous tubules (Lin et al., 2012). Historically, these cells had been identified as 'pre-spermatogonia' that exhibit the morphological features corresponding to those of human gonocytes

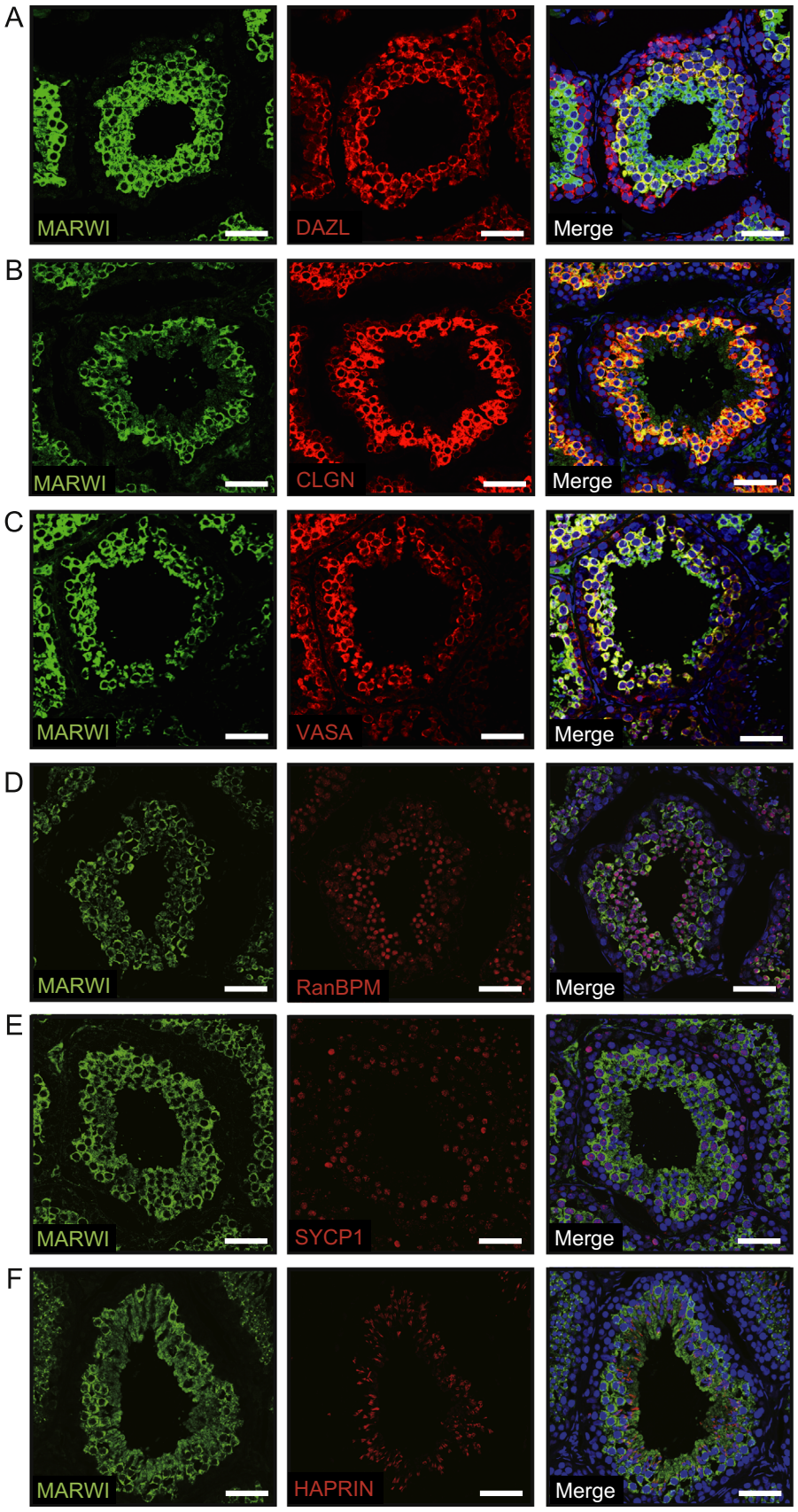

Fig. 3. Stage-specific expression of spermatogenesis-related proteins in adult marmoset testis. Confocal images of adult marmoset testis sections co-stained with antibodies against spermatogenesis-related proteins, including (A) anti-MARWI and anti-DAZL antibodies, (B) anti-MARWI and anti-CLGN antibodies, (C) anti-MARWI and anti-VASA antibodies, (D) anti-MARWI and anti-RanBPM antibodies, (E) anti-MARWI and anti-SYCP1 antibodies, and (F) anti-MARWI and anti-HAPRIN antibodies. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

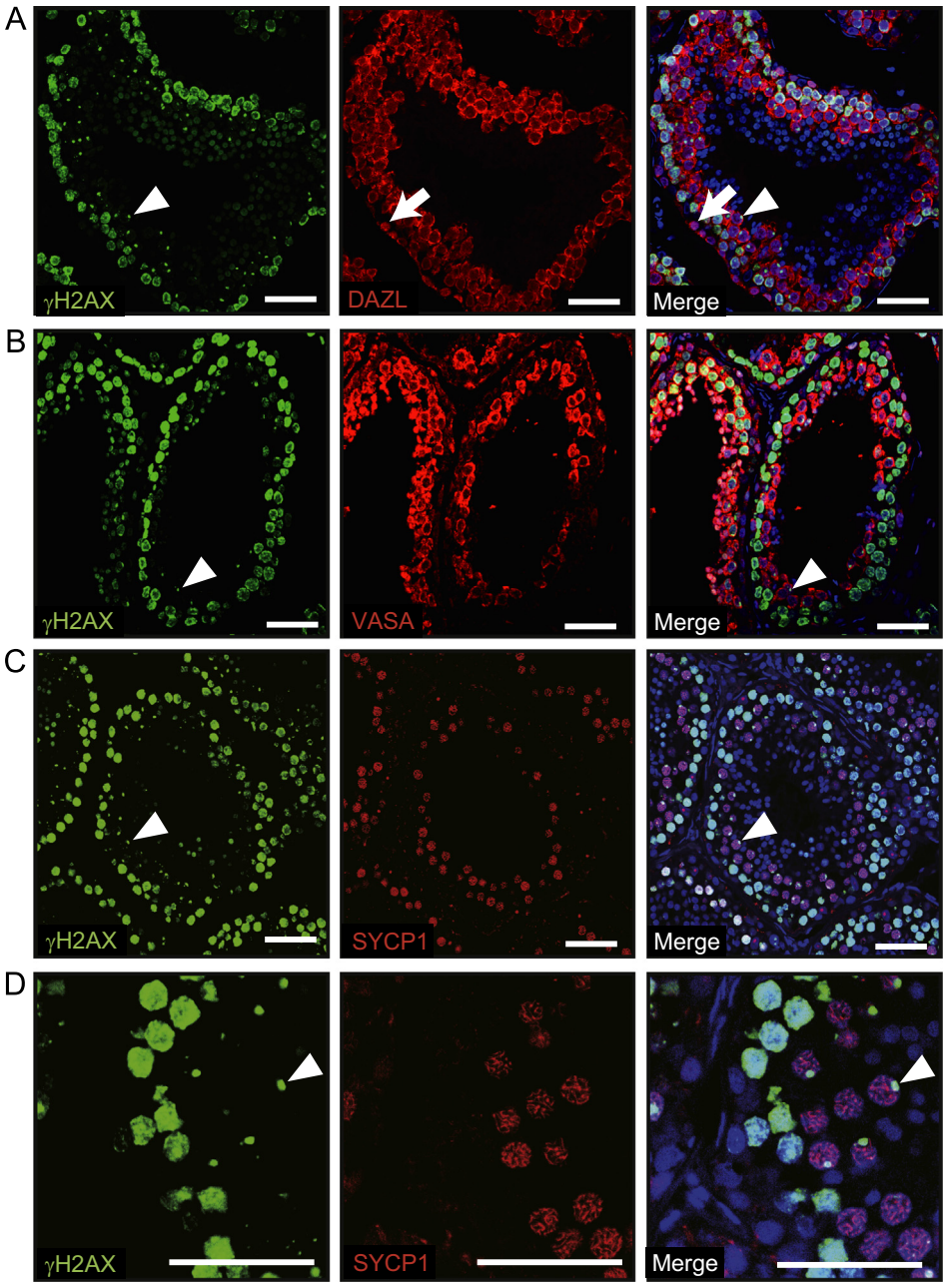

Fig. 4. Meiosis-associated formation of XY body and synaptonemal complex in the adult spermatocytes. Confocal images of adult marmoset testis sections co-stained with antibodies against germ cell and meiotic nuclear markers, including (A) anti-γH2AX and anti-DAZL antibodies, (B) anti-γH2AX and anti-VASA antibodies, and (C, D) anti-γH2AX and anti-SYCP1 antibodies. (D) A higher magnification of (C) clearly displays the formation of the XY body and synaptonemal complex in the nuclei of pachytene spermatocytes. Arrowheads indicate the XY body with confined sublocalization of γH2AX protein. In the image of (A), an arrow indicates DAZL^{+}γH2AX^{-} spermatogonia. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

but are consistently degenerated in the central part of seminiferous tubules (Li et al., 2005). Therefore, we next moved on to characterize the juvenile marmoset testis, particularly focusing on the pre-spermatogonia. According to our previous study, DAZL and VASA proteins were expressed in the pre-spermatogonia in 10-month juvenile marmoset testes whereas spermatogonia adjacent to the basement membrane expressed DAZL but not VASA protein (Fig. 5A and B). Surprisingly, the pre-spermatogonia also expressed MARWI protein, which in the adult was observed in the pachytene spermatocytes and onward (Fig. 3). Neither quiescent (SALL4) nor dividing spermatogonia (Ki67) markers were expressed in the MARWI^{+} pre-spermatogonia (Fig. 5C and D). Nevertheless, we sometimes found the pre-spermatogonia expressing SALL4 and C-KIT proteins (Fig. 5E) but they were negative for MARWI protein (Fig. 5C). These observations implied that the pre-spermatogonia might be a cell population that escapes from the normal commitment to a spermatogonial cell fate.

Then, we examined the expression of other meiosis-associated genes in the pre-spermatogonia. The MARWI^{+} pre-spermatogonia indeed expressed CLGN and RanBPM proteins (Fig. 6A and B), but the subcellular localization of RanBPM protein was confined to the cytoplasm unlike the nucleocytoplasmic localization observed in adults (Fig. 3D). We further investigated the meiotic nuclear structures based on the expression of γH2AX and SYCP1 proteins. Again, γH2AX was present in the VASA^{+} pre-spermatogonia (Fig. 6C–E). The nuclear distribution was primarily punctate (Fig. 6C) and only a few pre-spermatogonia exhibited the formation of an XY body (Fig. 6D). This was also the case in another juvenile marmoset testis with a slightly

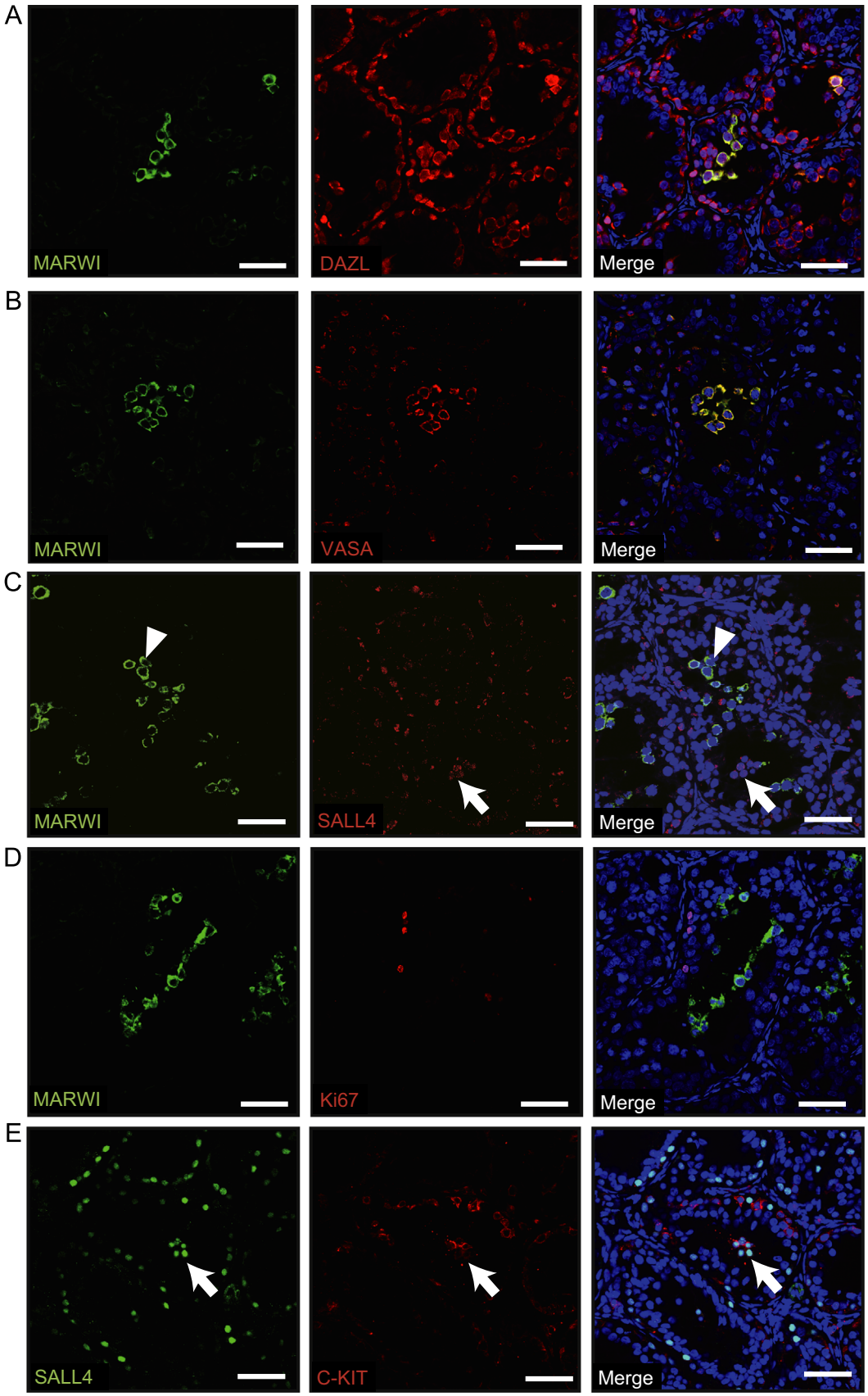

Fig. 5. Premature expression of MARWI protein in the pre-spermatogonia of juvenile marmosets. Confocal images of juvenile marmoset testis sections co-stained with (A) anti-MARWI and anti-DAZL antibodies, (B) anti-MARWI and anti-VASA antibodies, (C) anti-MARWI and anti-SALL4 antibodies, (D) anti-MARWI and anti-Ki67 antibodies, and (E) anti-SALL4 and anti-C-KIT antibodies. (C) SALL4 protein was observed in some pre-spermatogonia as well as spermatogonia population present on the basement membrane. The SALL4^{+} pre-spermatogonia were negative for MARWI (arrow) and vice versa (arrowhead). (E) A few pre-spermatogonia expressed both SALL4 and C-KIT proteins (arrow), while, in spermatogonia, these proteins were expressed in exclusive cell populations. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

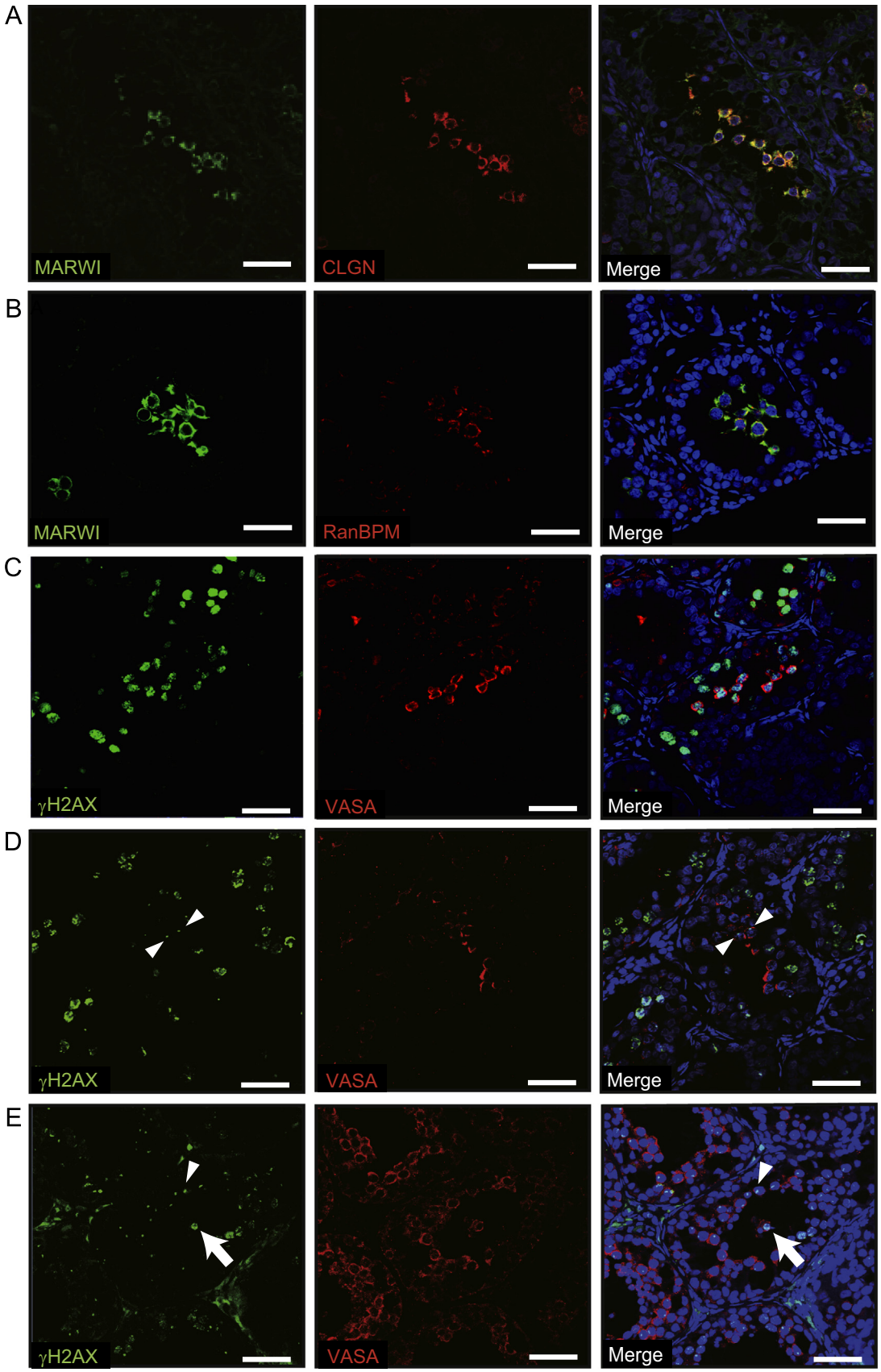

Fig. 6. Expression of meiotic markers in the pre-spermatogonia of juvenile marmosets. Confocal images of juvenile marmoset testis sections co-stained with antibodies against meiosis-associated proteins, including (A) anti-MARWI and anti-CLGN antibodies, (B) anti-MARWI and anti-RanBPM antibodies, and (C–E) anti-γH2AX and anti-VASA antibodies. (D) In a few seminiferous tubules, the $VASA^{+}$ pre-spermatogonia exhibited a γH2AX signal in the form of the XY body (arrowhead). (E) In the slightly mature juvenile testis, very few pre-spermatogonia remained, expressing VASA protein and γH2AX with the punctate nuclear distribution (arrow). The newly formed spermatocytes also expressed VASA protein and γH2AX but in the form of the XY body (arrowhead). Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

mature appearance, in which the number of the pre-spermatogonia had decreased (Fig. 6E). In the nuclei of the pre-spermatogonia, SYCP1 protein was also detectable in the form of a synaptonemal complex (Fig. 7A and B) but the abundance seemed much lower compared with adult spermatocytes (Fig. 4C and D). Additionally, in the juvenile testis of another marmoset with a slightly mature appearance, the synaptonemal complex seemed to be deformed in association with abnormal cellular and nuclear morphologies (Fig. 7C and D).

The pre-spermatogonia in juvenile marmoset testis undergo apoptosis

Given the observation of the degrading nuclear structure and cell morphology, we assumed that the pre-spermatogonia in juvenile testis might result in apoptosis. We therefore investigated apoptosis markers in the pre-spermatogonia, and found that activated Caspase3 (Said et al., 2004) was present in the pre-spermatogonia (Fig. 8A) and became predominant according to testicular development (Fig. 8B). The activated caspase3 was also observed in the spermatocytes derived from spermatogonia (Fig. 8B). We subsequently examined the cleavage form of PARP1, a target protein of activated Caspase3 in

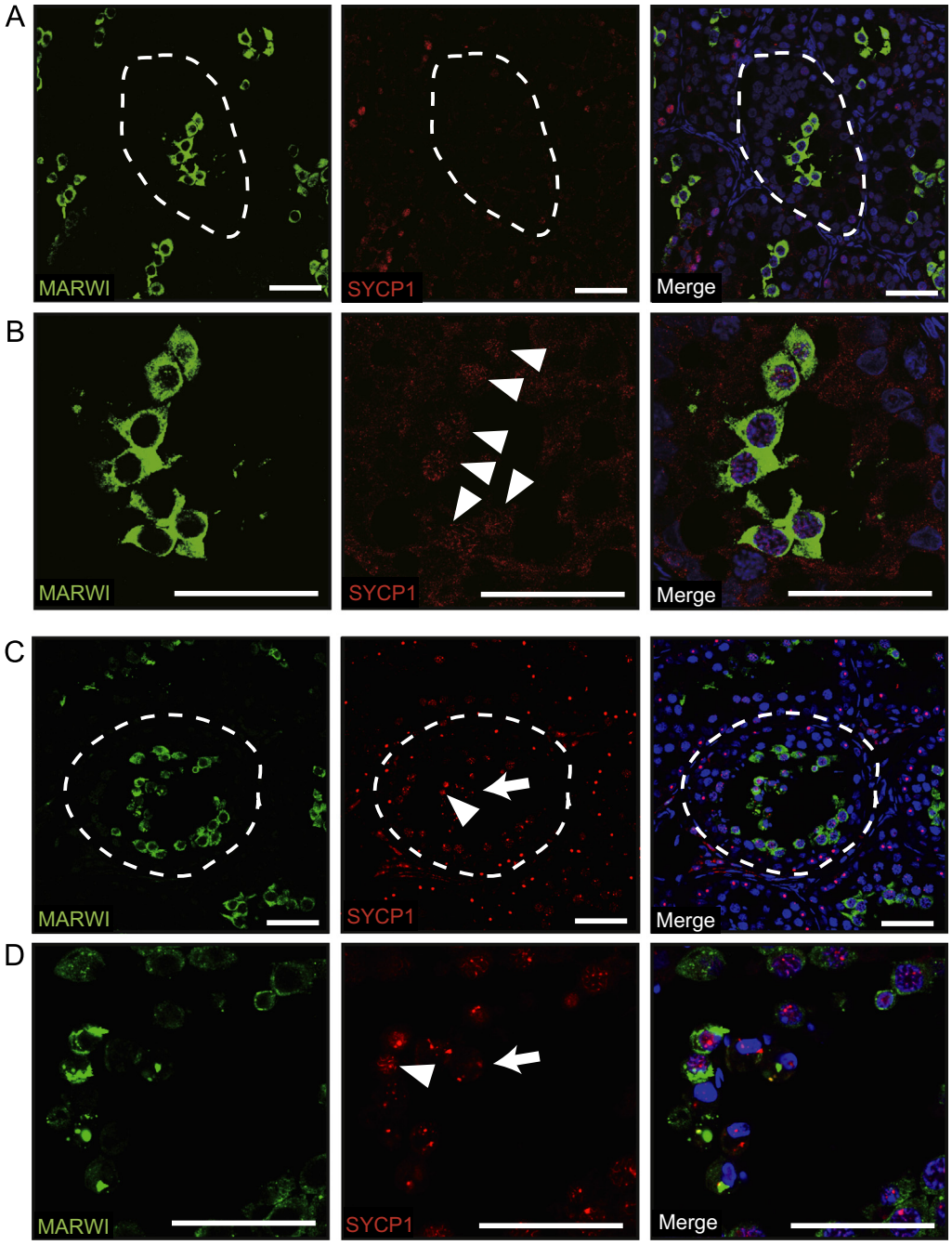

Fig. 7. Degeneration of synaptonemal complex in the pre-spermatogonia of juvenile marmosets. Confocal images of juvenile marmoset testis sections co-stained with anti-MARWI and anti-SYCP1 antibodies. (A, B) Immature juvenile testis. (B) A higher magnification of (A). Synaptonemal complex-like structures could be seen in the nucleus of the MARWI^{+} pre-spermatogonia (arrowhead) although the signal intensity was weaker than that observed in adult spermatocytes. (C, D) Juvenile marmoset testis with slightly mature appearance. (D) A higher magnification of (C). Some pre-spermatogonia formed synaptonemal complexes (arrowhead) while the others exhibited degenerated structures (arrow). Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

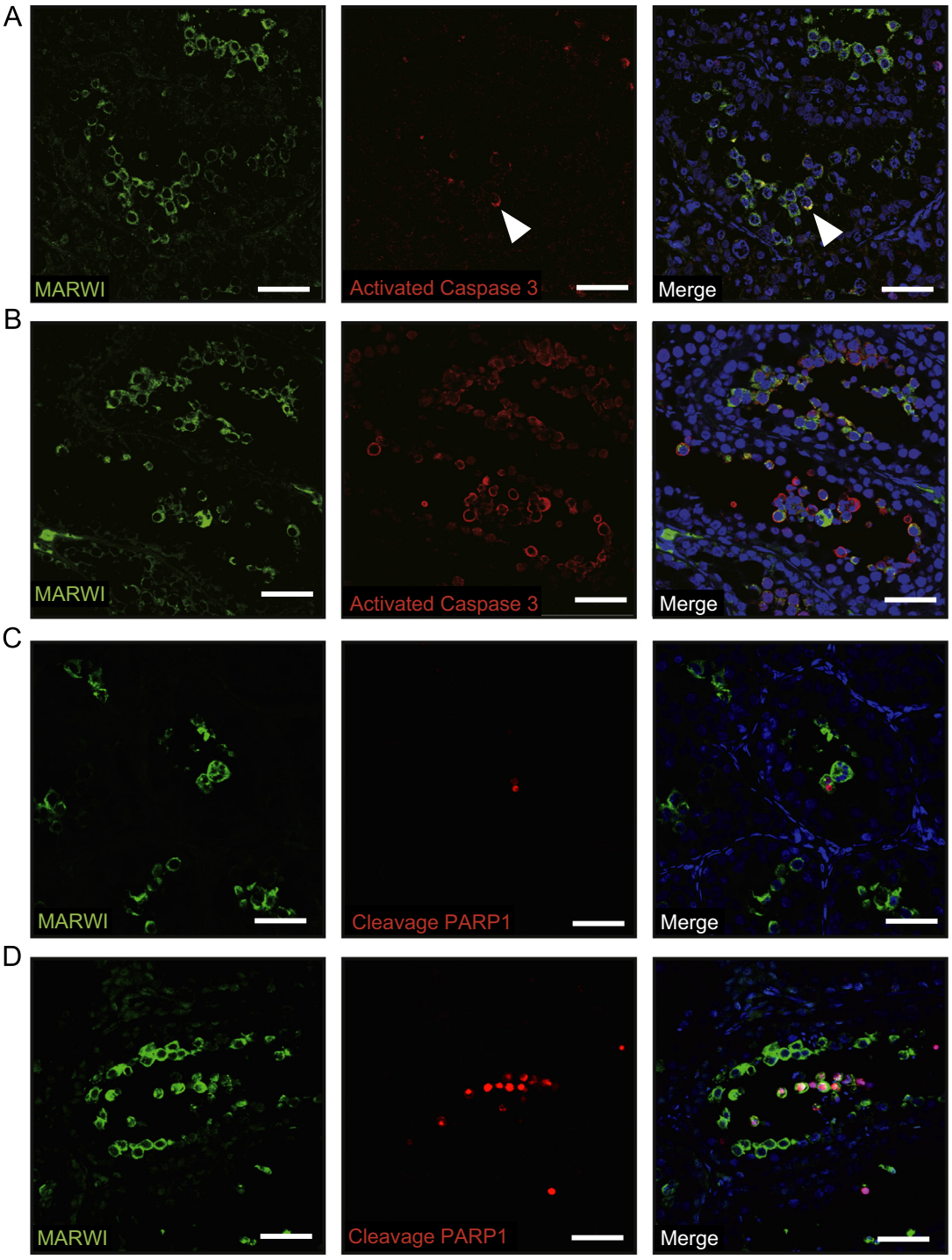

Fig. 8. Apoptotic markers in the pre-spermatogonia of juvenile marmosets. Confocal images of juvenile marmoset testis sections co-stained with antibodies against germ cell and apoptotic markers, including (A, B) anti-MARWI and anti-activated Caspase3 antibodies, and (C, D) anti-MARWI and anti-cleavage PARP1 antibodies. (A) In immature juvenile testis, a few pre-spermatogonia exhibited an activated Caspase3 signal (arrowhead). (B) Almost all of the pre-spermatogonia became positive for activated Caspase3 in juvenile testis with a slightly mature appearance. (C) In the immature juvenile testes, cleavage PARP1 was present in very few pre-spermatogonia. (D) In the juvenile testis with a slightly mature appearance, cleavage PARP1 was present in almost all of the pre-spermatogonia. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

an apoptotic pathway (YDBIO|6681Agarwal et al., 2009; D'Amours et al., 2001). A small number of pre-spermatogonia were positive for cleavage PARP1 in the less mature juvenile testis, similar to the case of activated caspase3 (Fig. 8C). In contrast, in the juvenile testis with a slightly mature appearance, cleavage PARP1 was frequently observed in the pre-spermatogonia but not in the spermatocytes (Fig. 8D). These data demonstrated that the pre-spermatogonia expressing meiosis-associated genes would be destined to undergo apoptotic cell death eventually.

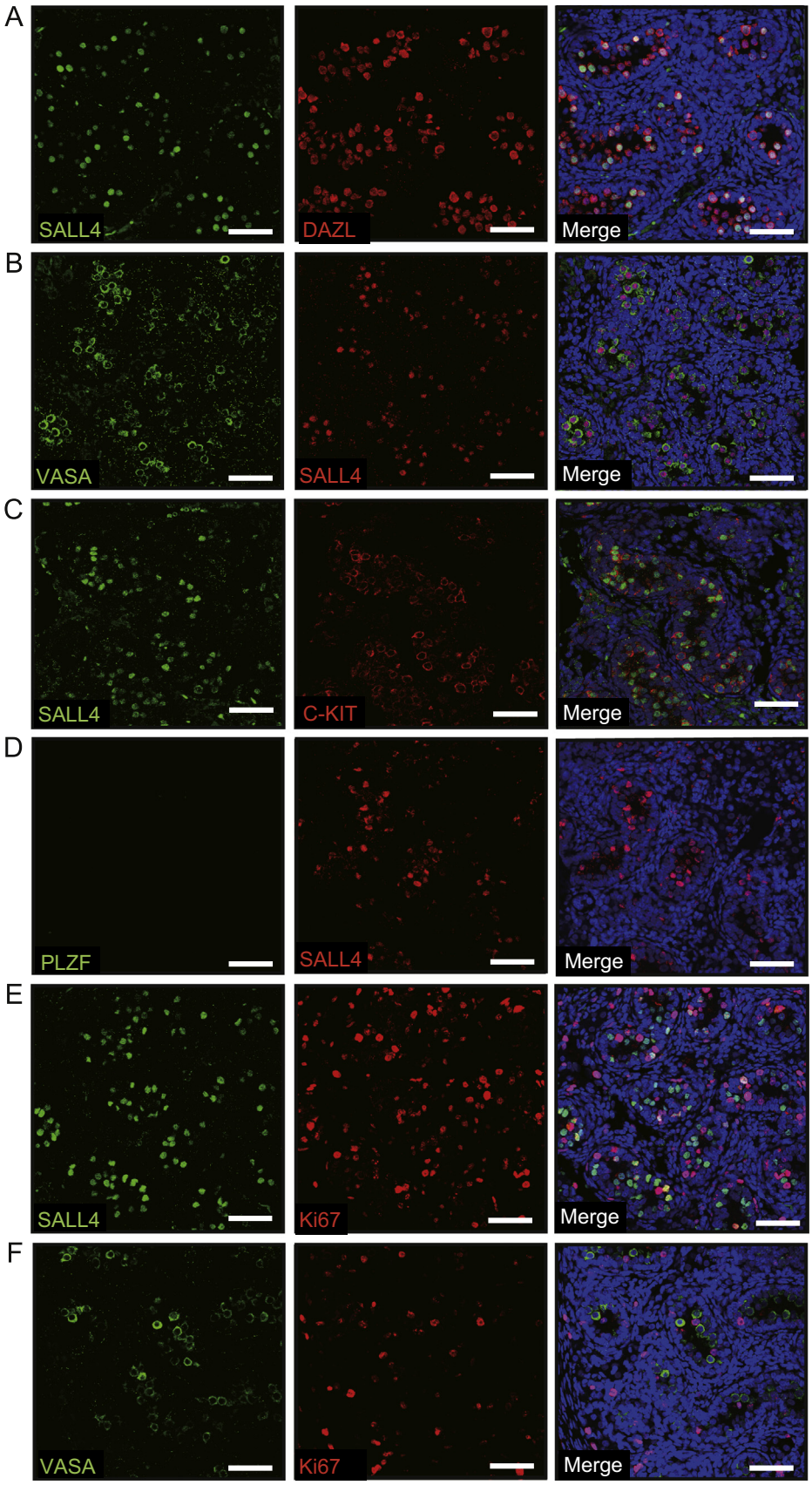

Fig. 9. Expression of germ cell markers in the neonatal gonocytes. Confocal images of neonatal marmoset testis sections co-stained with antibodies against germ cell markers, including (A) anti-SALL4 and anti-DAZL antibodies, (B) anti-VASA and anti-SALL4 antibodies, (C) anti-SALL4 and anti-C-KIT antibodies, (D) anti-PLZF and anti-SALL4 antibodies, (E) anti-SALL4 and anti-Ki67 antibodies, and (F) anti-VASA and anti-Ki67 antibodies. Nuclei were counterstained with Hoechst 33342. Scale bar, 50 μm.

The gonocyte origin of the pre-spermatogonia in juvenile marmoset testis

To identify whether the pre-spermatogonia in the juvenile testis originate from gonocytes or spermatogonia, we characterized the molecular signature of gonocytes in neonatal marmoset testis. Our previous study revealed that DAZL and VASA proteins were expressed in the marmoset gonocytes (Lin et al., 2012), similar to the pre-spermatogonia in juveniles. We found that the DAZL$^+$ VASA$^+$ gonocytes also expressed SALL4 protein (Fig. 9A and B) unlike the quiescent spermatogonia expressing SALL4 and DAZL but not VASA protein (YDBIO|6681Figs. 1 and 3) (Lin et al., 2012). Furthermore, in contrast to the spermatogonia, SALL4 and C-KIT proteins were co-expressed in the gonocytes (Fig. 9C) whereas the quiescent spermatogonia marker PLZF was absent (Fig. 9D), as is the case in mice (Hobbs et al., 2012). In addition, the SALL4$^+$VASA$^+$ gonocytes were Ki67-negative (Fig. 9E and F) as the neonatal gonocytes are mitotically arrested (YDBIO|6681Culty, 2009, 2013). Taken together, gene expression of gonocytes was very similar to that of the MARWI-negative population of pre-spermatogonia in juveniles (Fig. 5C and E). Considering the present results, the pre-spermatogonia most likely came from the gonocytes, but not spermatogonia, left over in the luminal region of seminiferous tubules during postnatal testicular development, and eventually disappear by apoptosis via a meiosis-like event.

Discussion

Developmental stages of primate spermatogenesis have been defined by classical cellular and nuclear morphologies and testis organization (YDBIO|6681Chemes, 2001; Dreef et al., 2007; Haruyama et al., 2012; Jackson and Edmunds, 1984; Millar et al., 2000; Schlatt and Ehmcke, 2014; Wistuba et al., 2003), while, in mice, the identification of numerous stage-specific marker genes has enabled each developmental process to be identified at a molecular level. By contrast only a limited number of molecular markers are available for primates. To develop our understanding of primate reproductive biology, it is fundamental and indispensable to expand molecular insights of primate spermatogenesis. In this study, we carried out sequential immunofluorescence analyses during postnatal testicular development in the common marmoset to depict a basic molecular atlas of spermatogenesis. Taken all together, we formulated the scenario of gene expression dynamics in germ cells during marmoset testicular development (Fig. 10). We believe that this insight could be essential for understanding primate germ cell development and cell culturing (YDBIO|6681Imamura et al., 2014, 2012).

According to Clermont's histological classification, primate undifferentiated spermatogonia are defined as two subtypes, mitotically quiescent (reserve) A_{dark} and actively dividing (renewing) A_{pale} spermatogonia (YDBIO|6681Clermont and Antar, 1973; Dym et al., 2009; Ehmcke and Schlatt, 2006; Millar et al., 2000). In the adult marmoset testis, we clearly identified two major mutually exclusive populations of spermatogonia, SALL4$^+$PLZF$^+$LIN28$^+$ DPPA4$^+$DAZL$^+$, and C-KIT$^+$Ki67$^+$DAZL$^+$ (YDBIO|6681Figs. 1, 10, and Supplementary Fig. S1). Considering that Ki67 is an indicator for mitotic division, it is conceivable that SALL4$^+$PLZF$^+$LIN28$^+$DPPA4$^+$ DAZL$^+$ quiescent cells would represent A_{dark} spermatogonia while C-KIT$^+$Ki67$^+$DAZL$^+$ active cells would represent A_{pale} spermatogonia, as defined by differential BrdU (YDBIO|6681Ehmcke et al., 2005a, 2005bSimorangkir et al., 2009) or ^{3}H-thymidine incorporation (Clermont, 1969). This scenario fits with the finding of human spermatogonia that FGFR3$^+$UTF1$^+$ A_{dark} and C-KIT$^+$Ki67$^+$ A_{pale} spermatogonia can be detected in a mutually exclusive manner (von Kopylow et al., 2012). However, the relationship between the A_{dark}/A_{pale} classification and our molecular discrimination should be carefully considered because the only faithful biological basis of the A_{dark}/A_{pale} classification is due to their mitotic activity. Classical discrimination of spermatogonial subtypes by hematoxylin staining largely relies on researchers' proficiency, hence, is methodologically inadequate for accurate validation and data comparison among laboratories (Ehmcke and Schlatt, 2006). The conclusion can differ depending on the protocols, equipment, and even observers from each experiment. Of course, there is no doubt that this classical classification provided an informative guideline to advance developmental biology of the primate spermatogenesis. However, this strategy has become insufficient to fulfill the current diagnostic criteria in the late-breaking stem cell biology for specifying a particular cell type. We determined the spermatogonial subtypes with easily accessible but specific immunofluorescence by focusing on their mitotic activity. This brings out unequivocal, reliable identification of spermatogonia in the common marmoset, leading to further molecular dissections.

On the other hand, the molecular character of differentiated B spermatogonia remains to be determined. In the current study, we found that C-KIT$^+$ spermatogonia are comprised of Ki67$^+$ and Ki67$^-$ cell subpopulations (Fig. 1B and Supplementary Fig. S2A). Furthermore, a part of C-KIT$^+$ spermatogonia exhibits the nucleus-diffuse, punctate γH2AX staining, as seen in the pre-pachytene spermatocytes (Supplementary Fig. S2B). Given that γH2AX protein is predominantly enriched in spermatocytes (Fig. 4) and the nucleus-diffuse γH2AX protein is also present in mouse B spermatogonia (Hamer et al., 2003), C-KIT$^+$Ki67$^-$DAZL$^+$γH2AX$^+$ spermatogonia might represent marmoset B spermatogonia.

At the onset of spermatogenesis, it seems that gene expression undergoes the sequential processes as follows (Fig. 10). The early spermatocytes derived from spermatogonia can be recognized as DAZL$^+$γH2AX$^+$CLGN^{weak+} cells, in which the γH2AX protein is widely distributed in the whole nucleus in a punctate manner. At the pachytene stage, spermatocytes become DAZL$^+$γH2AX$^+$CLGN$^+$VASA$^+$RanBPM$^+$SYCP1$^+$MARWI$^+$. In their nuclei, the γH2AX protein becomes confined to the XY body while SYCP1 protein localizes at the synaptonemal complexes. Then, round spermatids lose DAZL, γH2AX, and SYCP1 proteins but still express CLGN, VASA, RanBPM, and MARWI proteins. Finally, MARWI$^+$HAPRIN$^+$ elongating spermatids and spermatozoa appear. Thus, we describe the basic scheme of gene expression dynamics during marmoset spermatogenesis, but the information is still limited to a subset of genes. Nevertheless greater exploration of primate spermatogenesis is needed.

In addition to adult spermatogenesis, we also investigated gene expression dynamics in the sexually immature period before puberty. In the neonatal testis, gonocytes express SALL4 and C-KIT as well as DAZL and VASA proteins (Fig. 9) (Lin et al., 2012). During juvenile development, the gonocytes migrate to the basal membrane (McKinnell et al., 2013) where they form the SALL4$^+$ or C-KIT$^+$ spermatogonia. It is uncertain whether both types of spermatogonia are directly derived from the gonocytes. Considering gene expression in the adult spermatogonia, SALL4$^+$C-KIT$^+$DAZL$^+$VASA$^+$ gonocytes would turn to SALL4$^+$DAZL$^+$ spermatogonia while losing C-KIT and VASA expression. The SALL4$^+$DAZL$^+$ spermatogonia would subsequently give rise to C-KIT$^+$DAZL$^+$ spermatogonia.

On the other hand, some DAZL$^+$VASA$^+$ germ cells remain in the lumen of the juvenile seminiferous tubule (Fig. 5) (Lin et al., 2012). Previously, two types of germ cells could be observed in prepubertal marmoset testis; one in a central tubular position and the other at the basement membrane (Li et al., 2005). The identity of the centrally located germ cells, named pre-spermatogonia, had

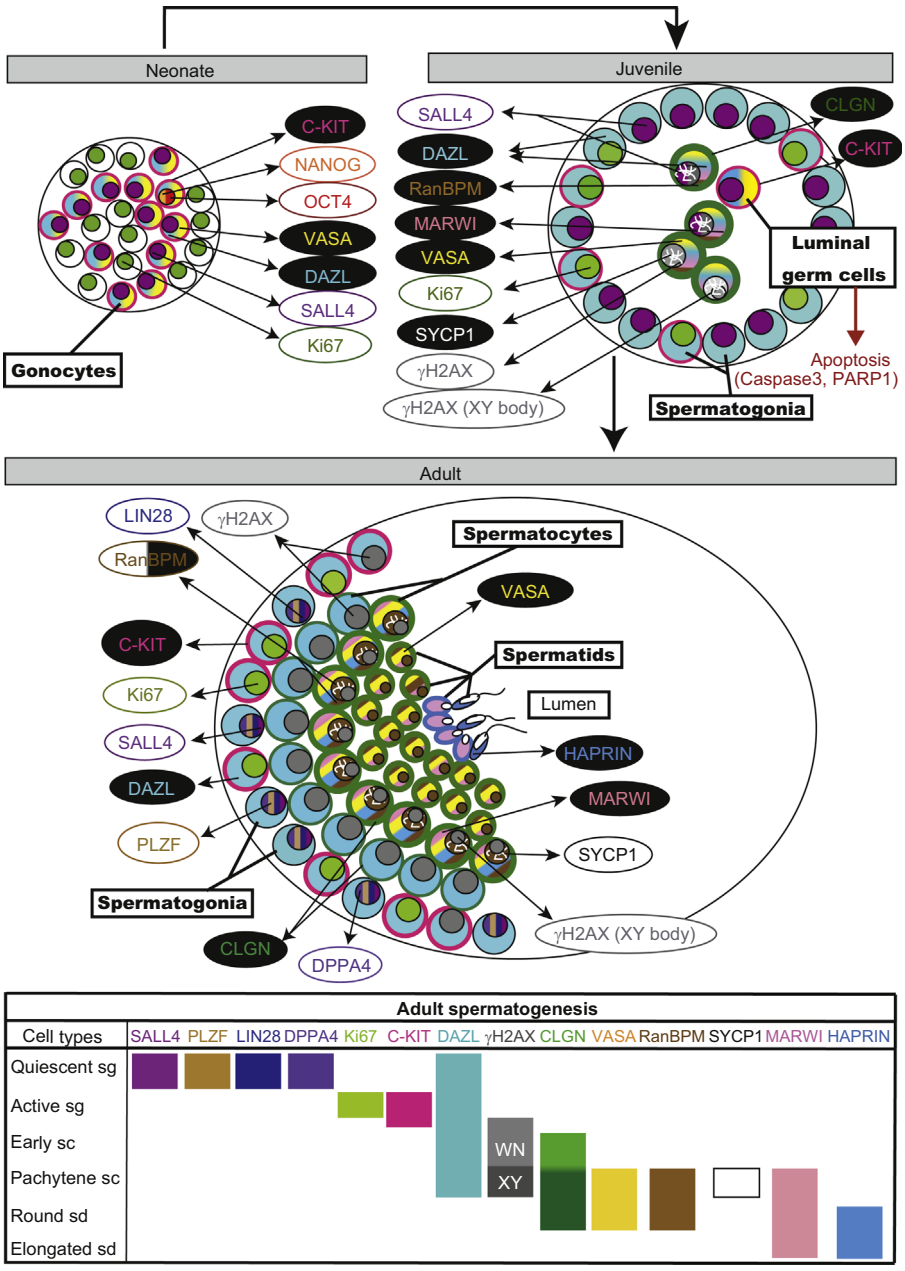

Fig. 10. A scheme of gene expression dynamics in the marmoset spermatogenesis. Schematic representation of spermatogenic development from neonate to adult in the common marmoset. In the seminiferous cords of neonatal testis, gonocytes express SALL4, C-KIT, DAZL, and VASA proteins. Pluripotency factors OCT4 and NANOG can be also observed in some of the gonocytes (Lin et al., 2012). Ki67 is present in non-germ cells, possibly Sertoli cell precursors. In the juvenile period, gonocytes relocated to the basement membrane turn to the member of the spermatogonial population. However, some gonocytes are left in the luminal region of seminiferous tubules. They lose the gonocyte gene expression and exhibit the spermatocyte-like expression, including DAZL, γH2AX, CLGN, VASA, RanBPM (cytoplasmic), SYCP1, MARWI proteins as well as an apoptosis marker (activated Caspase3, cleavage PARP1). In adult testis, SALL4$^+$PLZF$^+$LIN28$^+$DPPA4$^+$DAZL$^+$ quiescent spermatogonia (Quiescent sg) and C-KIT$^+$Ki67$^+$DAZL$^+$ active spermatogonia (Active sg) can be seen on the basement membrane. Upon differentiation, early spermatocytes (early sc) are recognized as DAZL$^+$γH2AX$^+$CLGN^{weak+} cells. At this stage, the distribution of γH2AX protein is in the whole nucleus (WN). Then, pachytene spermatocytes (pachytene sc) turn to be DAZL$^+$γH2AX$^+$CLGN$^+$VASA$^+$RanBPM$^+$SYCP1$^+$MARWI$^+$. The γH2AX protein is confined to the XY body (XY). Round spermatids (round sd) still express CLGN, VASA, RanBPM, and MARWI proteins. Finally, MARWI$^+$HAPRIN$^+$ elongating and elongated spermatids (elongated sd) and spermatozoa appear.

not been elucidated, but it turned out that these cells exhibited meiosis-associated gene expression. Nevertheless, the meiotic process seemed irregular, and the pre-spermatogonia result in apoptosis. In general, during development, germ cells should be recruited to the appropriate niches. If primordial germ cells are misdirected from the normal path, they undergo apoptosis (Tres et al., 2004). Likewise, migration failure to the basement membrane induces apoptosis in mouse gonocytes (YDBIO|6681Orth et al., 2000; Tres and Kierszenbaum, 2005). Taking this into account, we speculate that a part of the marmoset gonocytes might fail to migrate to the basement membrane and be eliminated by apoptosis through a meiosis-like process in the juvenile period. This scenario is also supported by cytoplasmic localization of RanBPM protein observed in pre-spermatogonia, which indicates the activation of an apoptotic pathway (Atabakhsh et al., 2009). However, we can still not exclude another possibility that the pre-spermatogonia might be abnormal spermatocytes derived from the start-up spermatogonia. In this assumption, the pre-spermatogonia (spermatocytes) might be located ectopically in the luminal region of the seminiferous tubules because of

immaturity of testicular niche and hormonal regulation (Li et al., 2005), which cannot support meiosis properly. In order to determine the origin of the pre-spermatogonia, further comprehensive, serial dissection of gene expression dynamics focusing on the neonatal-juvenile period is needed.

Following testicular development, juvenile spermatogonia start producing spermatocytes (YDBIO|6681Figs. 6 and 7), most of which are positive for activated Caspase3 (Fig. 8). Since the first wave of spermatogenesis mobilized after birth is accompanied by massive apoptosis of spermatocytes in rodents (Rodriguez et al., 1997), this could also occur in the common marmoset. On the other hand, unlike the pre-spermatogonia, cleavage PARP1 was not observed in the spermatocytes (Fig. 8). This could be explained by the different time course of apoptosis between pre-spermatogonia and spermatocytes. The pre-spermatogonia appeared earlier than the spermatocytes did during testicular development. Indeed, many activated caspase3- and cleavage PARP1-negative pre-spermatogonia existed in the less mature juvenile testis, in which the spermatocytes did not appear (Fig. 8). As the cleavage of PARP1 is implemented by the activated caspase3 (YDBIO|6681Agarwal et al., 2009; D'Amours et al., 2001; Said et al., 2004), the spermatocytes may become positive for cleavage PARP1 in later development.

In conclusion, we investigated molecular signatures of germ cells during postnatal testicular development in the common marmoset, which defined gene expression involved with developmental transitions. This also clarified, at least partly, the characteristics of primate germ cells, especially in the juvenile period. We expect that this fundamental ontogeny promotes better understanding of the primate germ cells and reproduction thereafter.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.01.014.

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