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ORIGINAL ARTICLE

Molecular histology of spermatogenesis in the Japanese macaque monkey (*Macaca fuscata*)

Sawako Okada[1] · Kota Kuroki[1] · Cody A. Ruiz[2,3] · Anthony J. Tosi[2,3] · Masanori Imamura[1]

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Abstract

Non-human primates are our closest relatives and therefore offer valuable comparative models for human evolutionary studies and biomedical research. As such, Japanese macaques (*Macaca fuscata*) have contributed to the advancement of primatology in both field and laboratory settings. Specifically, Japanese macaques serve as an excellent model for investigating postnatal development and seasonal breeding in primates because of their relatively prolonged juvenile period and distinct seasonal breeding activity in adulthood. Pioneering histological studies have examined the developmental associations between their reproductive states and spermatogenesis by morphological observation. However, a molecular histological atlas of Japanese macaque spermatogenesis is only in its infancy, limiting our understanding of spermatogenesis ontogeny related to their reproductive changes. Here, we performed immunofluorescence analyses of spermatogenesis in Japanese macaque testes to determine the expression of a subset of marker proteins. The present molecular histological analyses readily specified major spermatogonial subtypes as SALL4$^+$ A spermatogonia and Ki67$^+$/C-KIT$^+$ B spermatogonia. The expression of DAZL, SCP1, γH2AX, VASA, and calmegin further showed sequential changes regarding the protein expression profile and chromosomal structures during spermatogenesis in a differentiation stage-specific manner. Accordingly, comparative analyses between subadults and adults identified spermatogenic deficits in differentiation and synchronization in subadult testes. Our findings provide a new diagnostic platform for dissecting spermatogenic status and reproduction in the Japanese macaques.

Keywords Japanese macaque · Spermatogenesis · Immunohistology

Introduction

The Japanese macaque (*Macaca fuscata*) is the most northern-living nonhuman primate species. Extensive field and laboratory studies of this species have yielded significant contributions to the advancement of primatology. Field researchers have investigated the ecology and social behaviour of the Japanese macaque (Leca et al. 2016; Schofield et al. 2018; Takahata et al. 1999), while life scientists, especially neuroscientists, have focused on biomedical aspects, such as their anatomical and physiological similarities to humans, their high cognitive ability, and their unusually tolerant social behaviour (Iriki and Sakura 2008; Isa et al. 2009). In addition, the Japanese macaque exhibits unique reproductive characteristics compared to other commonly studied model organisms, such as mice and rats. For example, like other primates, they undergo a relatively prolonged juvenile period (4–5 years in the Japanese macaque) before reaching sexual maturity. They also have seasonal breeding activity adapted to distinct annual climate changes in the Japanese islands. Thus, the Japanese macaque is an excellent model for investigating postnatal development and seasonal breeding in primates.

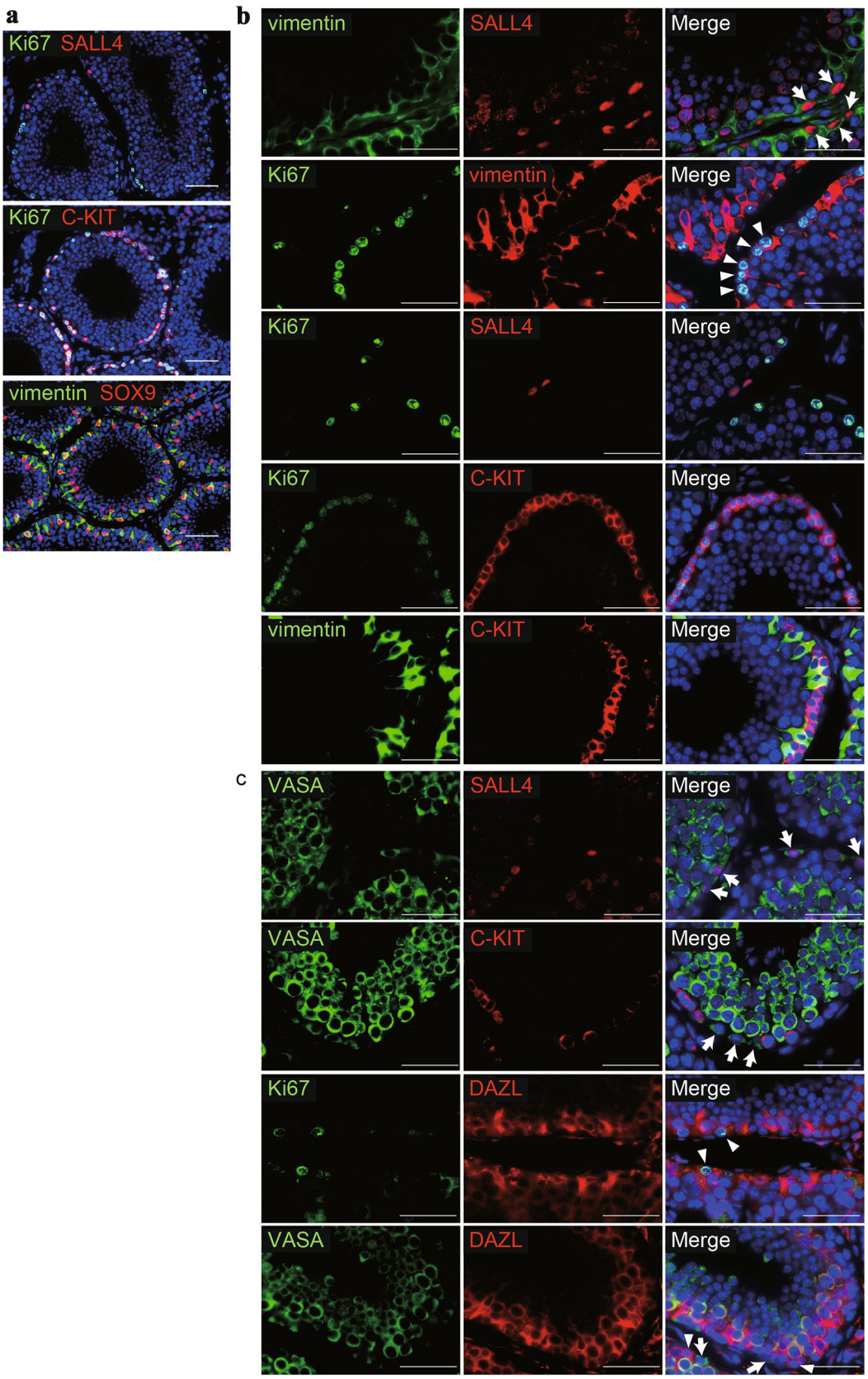
In mammals, spermatogenesis occurs in seminiferous tubules of adult testis. This sequential process is spatiotemporally regulated under a strict developmental program (Hermo et al. 2010). Spermatogenesis originates from spermatogonia, which are enclosed by Sertoli cells on the basement membrane of seminiferous tubules. Spermatogonia either renew themselves by mitotic division or proceed to

✉ Masanori Imamura
imamura.masanori.2m@kyoto-u.ac.jp

[1] Molecular Biology Section, Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan

[2] Department of Anthropology, Kent State University, Kent, Ohio, USA

[3] School of Biomedical Sciences, Kent State University, Kent, Ohio, USA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◂**Fig. 1** Spermatogonial subtypes in the adult Japanese macaque testis. Testis sections from adult Japanese macaques (*Mf2338*, 93 months old; *Mf2348*, 154 months old) were characterized by immunofluorescence analyses with specific antibodies. Representative images of each immunofluorescence staining are presented. **a** Immunofluorescence images of spermatogonia (SALL4, Ki67, C-KIT) and Sertoli cell (vimentin, SOX9) distribution. **b** High-magnification images of undifferentiated A spermatogonia (SALL4), differentiating spermatogonia (Ki67, C-KIT), and Sertoli cells (vimentin). **c** High-magnification images of spermatogonia expressing VASA and DAZL proteins. Arrows indicate A spermatogonia, and arrowheads indicate B spermatogonia. Nuclei were counterstained with DAPI. Scale bar: 50 µm

meiosis to produce spermatocytes, spermatids, and finally mature spermatozoa. The spermatogenic process becomes active after sexual maturation. In neonates, testicular structures are still under construction, and only gonocytes, a spermatogonia progenitor, exist in seminiferous cords. Spermatogenesis enters the preparatory stage in juveniles, and full spermatogenesis is achieved in adulthood. Regarding the Japanese macaque, pioneering studies have characterized the morphology of spermatogenic cells by classical histological strategies, such as hematoxylin–eosin (HE) or periodic acid-Schiff (PAS) staining (Enomoto et al. 1994, 1995; Nagato et al. 1994). These observations also implied the presence of seasonal changes in spermatogenesis, which include less spermatogenic activity (Enomoto et al. 1994) and degeneration of spermatogenic cells (Enomoto et al. 1995) in the non-mating season. However, only a few subsequent studies have targeted precise molecular characterization of spermatogenesis based on cell type-specific antigens (Tokunaga et al. 1999; Yu and Takenaka 2004). Consequently, a molecular histological atlas of Japanese macaque spermatogenesis has not yet been established, limiting our understanding of spermatogenesis ontogeny and reproductive signatures in this species.

In the present study, we performed immunofluorescence analyses of Japanese macaque testes in order to characterize spermatogenic cells by molecular criteria. We identified spermatogonia subtypes and the developmental sequence of spermatogenesis, which can help assess subadult-adult spermatogenic status. These results provide an essential platform for expanding our understanding of Japanese macaque reproduction.

Materials and methods

Tissues and ethics

All experiments in this study were approved by the Animal Care and Use Committee of Kyoto University Primate Research Institute (KUPRI) and were performed in accordance with the Guidelines for Care and Use of Nonhuman Primates (version 3, 2010) published by KUPRI. For tissue collection, testes were obtained from four Japanese macaques (*Mf2348*, 2005/5/16 birth, 2018/3/16 euthanasia; *Mf2338*, 2009/6/26 birth, 2017/3/29 euthanasia; *Mf2431*, 2011/5/23 birth, 2017/4/11 euthanasia; *Mf2433*, 2011/5/26 birth, 2016/12/28 euthanasia) that were euthanized because of other experiments, but not for this study. All four monkeys had no history of producing offspring, because they had been housed individually. However, they had no medical record of diseases or accidents which might have affected their reproductive growth or abilities.

Histological analyses

Each testis was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax, and sectioned at 5 µm thickness. Testis sectioning and HE staining were performed by BioGate Co. (Gifu, Japan). For immunofluorescence analyses, the sections were de-waxed with G-NOX (GenoStaff, GN04), rehydrated in a graded alcohol series, and washed with distilled water. The sections were subjected to antigen retrieval at 110 °C for 15 min in 1× Target Retrieval Solution (Dako, S1699). They were then treated with 0.1% Triton-X, blocked with 5% skimmed milk, and incubated with primary antibodies at 4 °C overnight in a humidified chamber. The following primary antibodies were used: rabbit anti-SALL4 (1:100, Abcam, ab29112), rat anti-Ki67 (1:100, eBioscience, 14-5698-82), mouse anti-Vimentin (1:100, Abcam, ab8069), rabbit anti-Vimentin (1:100, Abcam, ab92547), rabbit anti-SOX9 (1:100, Abcam, ab185966), rabbit anti-C-KIT (1:100, Abcam, ab32363), mouse anti-DDX4 (VASA) (1:50, Abcam, ab27591), rabbit anti-DAZL (1:150, Abcam, ab34139), rabbit anti-SCP1 (1:100, Abcam, ab175191), mouse anti-phospho-histone H2A.X (ser139) (γH2AX) (1:100, Millipore, 05-636), and rabbit anti-Calmegin (1:100, Abcam, ab171971). Secondary antibodies included Alexa Fluor Plus 488 goat anti-mouse IgG (1:400, Invitrogen, A32723), Alexa Fluor 488 goat anti-rat IgG (1:500, Abcam, ab150157), and Alexa Fluor 555 goat anti-rabbit IgG (1:500, Invitrogen, A21429). Nuclei were counterstained with 1 µg/ml DAPI. For each experiment, a negative control was included in which the primary antibody had been omitted. Images were captured using a BZ-X700 fluorescence microscope (Keyence).

Results

Testes from two adult Japanese macaques (*Mf2338*, 93 months old; *Mf2348*, 154 months old) were subjected to molecular histological analyses of spermatogenesis. In primates, spermatogonia are grouped into two major subtypes: slowly cycling, undifferentiated A spermatogonia, and transit-amplifying, differentiating B spermatogonia

(Fayomi and Orwig 2018; Hermann et al. 2010). To identify the spermatogonial subtypes in the Japanese macaque, we performed immunofluorescence analysis of spermatogonia (SALL4, Ki67, C-KIT) and Sertoli cells (vimentin, SOX9) markers (Fig. 1a). SALL4 and Ki67 are molecular indicators of undifferentiated and dividing spermatogonia, respectively (Fayomi and Orwig 2018; Lin et al. 2015). Both SALL4$^+$ and Ki67$^+$ spermatogonia were enclosed by vimentin$^+$ Sertoli cells, but in a mutually exclusive manner (Fig. 1b). Oval SALL4$^+$ spermatogonia were present on the basement membrane and distributed sparsely, whereas larger round Ki67$^+$ spermatogonia were located close to the basement membrane with aligned distribution. The number of Ki67$^+$ spermatogonia was approximately 2.5-fold more than that of SALL4$^+$ spermatogonia. The Ki67$^+$ spermatogonia co-expressed a differentiating spermatogonia marker C-KIT (Fayomi and Orwig 2018). Almost half of the SALL4$^+$ spermatogonia were VASA$^+$/DAZL$^-$ while the Ki67$^+$/C-KIT$^+$ spermatogonia were VASA$^-$/DAZL$^+$ (Fig. 1c).

We next examined immunohistological characteristics from spermatocytes to spermatids. Previous morphological observations identified short pre-pachytene and long pachytene stages in spermatocytes (Nagato et al. 1994). As molecular indicators of the spermatocyte stages, we focused on two meiosis-associated chromosomal configurations: synaptonemal complexes for the autosomes (Page and Hawley 2004) and XY bodies for the sex chromosomes (de Vries et al. 2012). Immunostaining with SCP1 and γH2AX detected these two types of chromosomal configurations, respectively (Fig. 2a). Interestingly, the immunostaining patterns of SCP1 and γH2AX differed among spermatocyte stages (Fig. 2b). The pre-pachytene spermatocytes exhibited distribution of SCP1 at the nuclear margins, but did not show γH2AX signal indicative of XY body formation. Then, in the early pachytene spermatocytes, XY body formation could be detected (and SCP1 was still enriched at the nuclear margin). Synaptonemal complexes and XY bodies were both evident in the pachytene spermatocytes.

Following from the immunostaining patterns of SCP1 and γH2AX, we characterized developmental stage-specific expression of DAZL, VASA, and calmegin proteins (Fig. 3). A germ cell-specific RNA-binding protein DAZL was detected from spermatogonia to the pachytene spermatocytes, which co-expressed γH2AX in the XY body. In contrast, another RNA-binding protein VASA was expressed weakly in the early pachytene spermatocytes and strongly in the pachytene spermatocytes and round spermatids. Calmegin, a testis-specific chaperone protein, was only faintly detected in the early pachytene and pachytene spermatocytes and then yielded a more robust signal in round spermatids.

Finally, we attempted to assess developmental changes in spermatogenesis between subadult and adult monkeys by immunofluorescence analyses. Testes from two subadults (*Mf2433*, 67 months old; *Mf2431*, 71 months old), which lacked sperm in the seminiferous tubules, were compared with those from two adults (Fig. 4a). In the 67-month-old subadult testes, only a few DAZL$^+$/VASA$^+$ and VASA$^+$/calmegin$^+$ spermatocytes were detected (Fig. 4b). In the 71-month-old subadult, these spermatocytes were observed more frequently and DAZL$^-$ round spermatids appeared, but their distribution was uneven and spermatogenesis was not completed. These observations were quite distinct from those of synchronized full spermatogenesis in adults, which depict sequential protein expression changes. Thus, immunohistological kinetics can be used to evaluate spermatogenic stages and cell distribution reflecting the reproductive status of Japanese macaques.

Discussion

Here, we described the expression kinetics of a subset of antigens throughout spermatogenesis in the Japanese macaque (Fig. 5). Pioneering histological studies examined spermatogenesis in this species via morphological observation with HE or PAS staining. These efforts provided basic insights into seminiferous cycles (Nagato et al. 1994) and seasonal reproductive changes (Enomoto et al. 1994, 1995). However, classical histological methods relied largely on the researchers' proficiency—an approach which created difficulty in accurate validation, reproducibility, and technical convenience. In contrast, protein expression-based molecular dissection has enabled us to characterize the developmental properties of spermatogenesis with precision and detail. Our results indicate that the molecular ontogeny of the Japanese macaque spermatogenesis is essentially similar to that of the rhesus macaque (Fayomi and Orwig 2018; Sharma et al. 2017) and the common marmoset (Lin et al. 2015, 2012), suggesting an evolutionarily conserved molecular foundation for primate (haplorhine) spermatogenesis. Immunohistological kinetics thus provide a useful new platform for investigating spermatogenesis in the Japanese macaque, which is a prerequisite for investigating their postnatal development and seasonal reproduction.

Subtypes of primate spermatogonia have received much attention in studies of reproductive biology. Hematoxylin staining revealed two subcategories of primate type 'A' spermatogonia: A_{dark} and A_{pale} (Clermont and Leblond 1959). Based on in vivo labelling of mitotic cells, A_{dark} and A_{pale} were considered as reserve stem cells and active progenitors, respectively (Clermont 1969; Ehmcke and Schlatt 2006). However, recent studies argued that these two subtypes are identical cells at different cell cycle phases (Fayomi and Orwig 2018; Hermann et al. 2010). In the present study, we observed VASA$^+$ and VASA$^-$ A spermatogonia at nearly

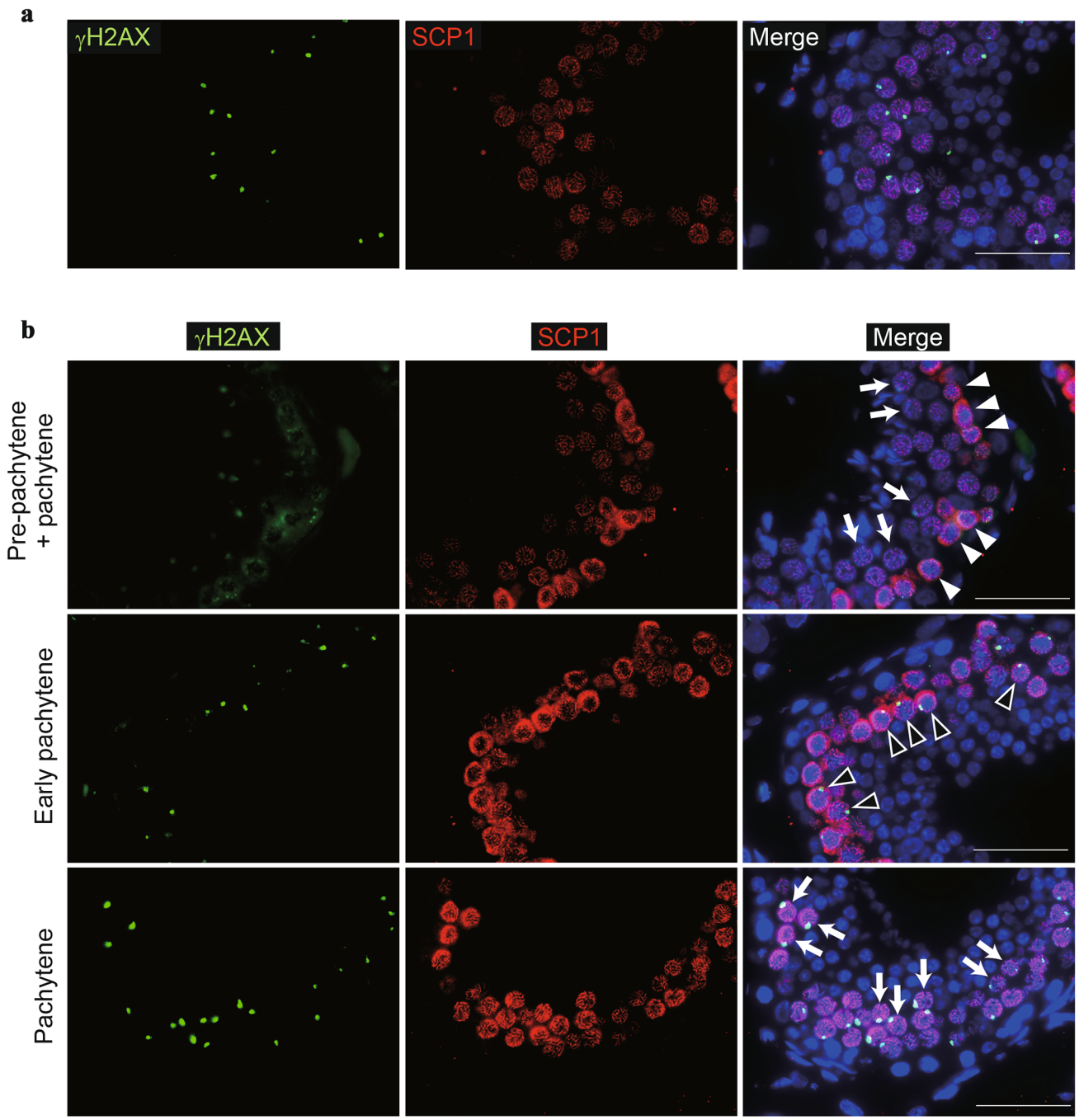

Fig. 2 Chromosome configuration-based classification of spermatocytes in the adult Japanese macaque. Testis sections from adult Japanese macaques (*Mf2338*, 93 months old; *Mf2348*, 154 months old) were characterized by immunofluorescence analyses with specific antibodies. Representative images of each immunofluorescence staining are presented. **a** Nuclear localization of γH2AX and SCP1 proteins in XY bodies and synaptonemal complexes, respectively. **b** Three spermatocyte stages represented by γH2AX and SCP1 immunostaining. The pre-pachytene spermatocytes (closed arrowheads) exhibited prominent localization of SCP1 at the nuclear margins, but no XY body formation of γH2AX. The early pachytene spermatocytes (open arrowheads) exhibited formation of XY bodies, as well as continued signal of SCP1 at the nuclear margins. The pachytene spermatocytes (arrows) exhibited synaptonemal complexes of SCP1 and XY bodies. The upper images show a section of seminiferous tubule including both the pre-pachytene and pachytene spermatocytes. Nuclei were counterstained with DAPI. Scale bar: 50 μm

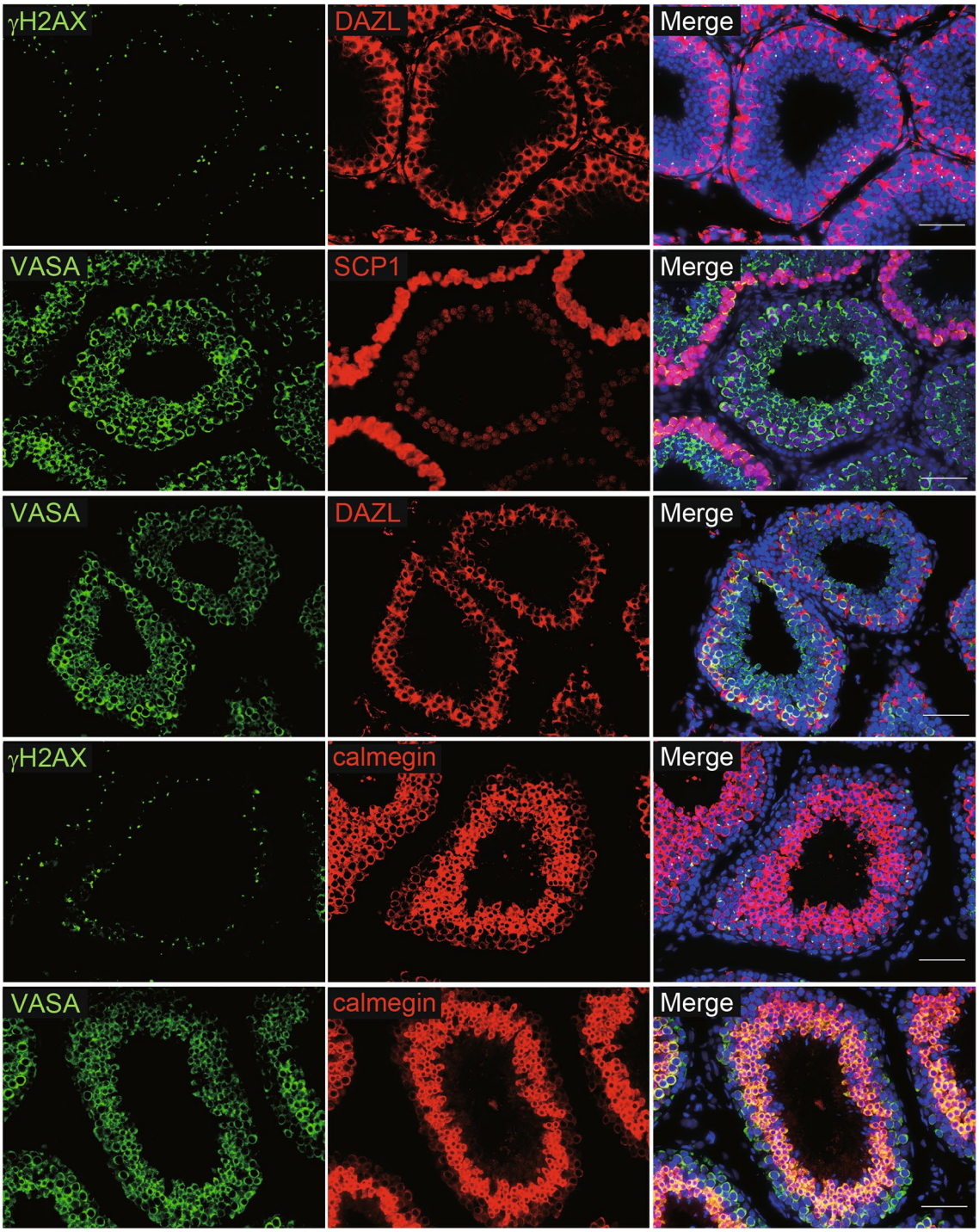

Fig. 3 Stage-specific expression of spermatogenesis-associated proteins in the adult Japanese macaque testis. Testis sections from adult Japanese macaques (*Mf2338*, 93 months old; *Mf2348*, 154 months old) were characterized by immunofluorescence analyses with specific antibodies against spermatogenesis-associated proteins (DAZL, VASA, SCP1, γH2AX, calmegin). Representative images of each immunofluorescence staining are presented. Nuclei were counterstained with DAPI. Scale bar: 50 μm

the same frequency (Fig. 1c). Considering that the numbers of A_{dark} and A_{pale} subtypes are likewise balanced (Marshall and Plant 1996), the $VASA^+$ and $VASA^-$ distinction is likely to be a simple reflection of these underlying subtypes, as previously demonstrated by immunostaining with PGP9.5 protein (Tokunaga et al. 1999). In addition, while $VASA^+$ A spermatogonia were $DAZL^-$, it remains to be seen whether $VASA^-$ A spermatogonia express DAZL (not determined

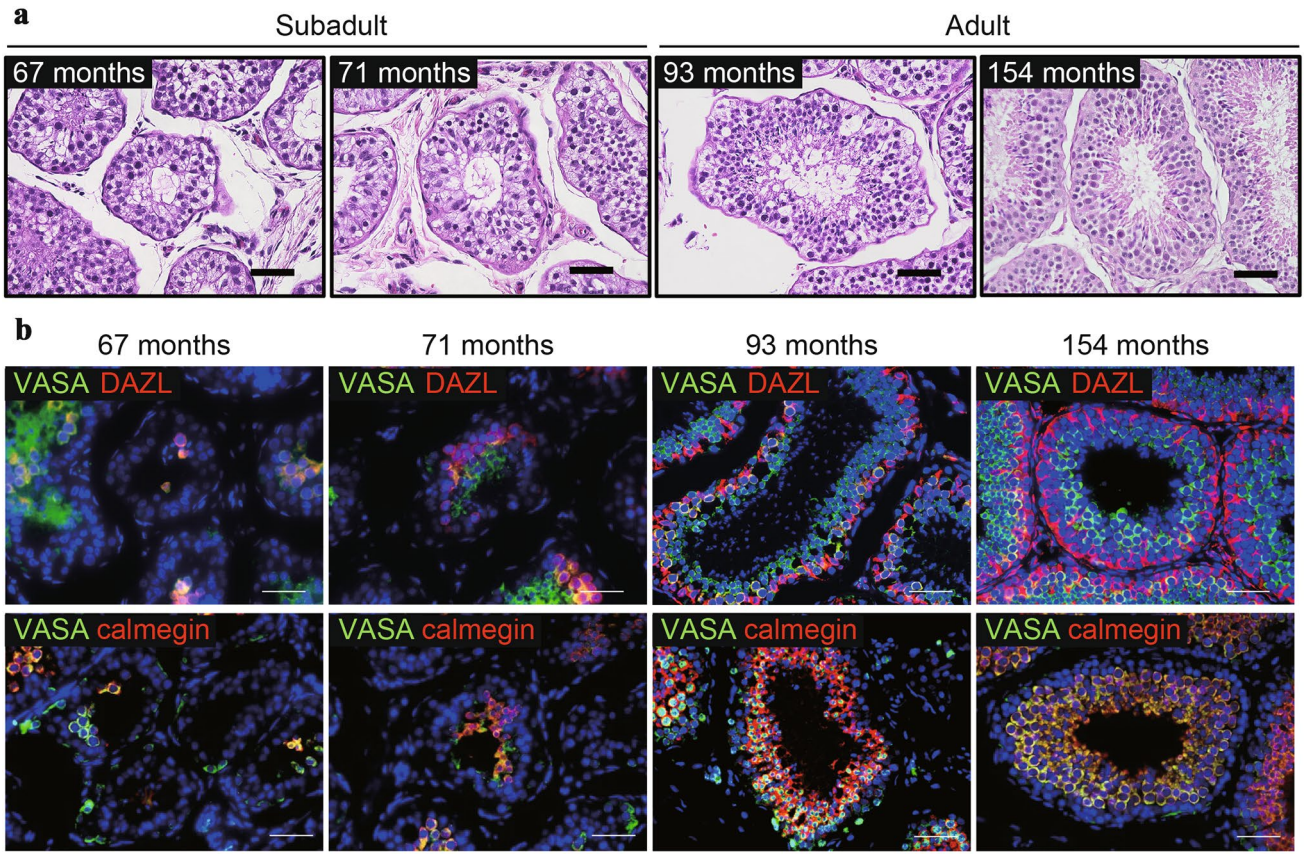

Fig. 4 Immunohistological differences in subadult-adult spermatogenic status. Testis sections from subadult (*Mf2433*, 67 months old; *Mf2431*, 71 months old) and adult (*Mf2338*, 93 months old; *Mf2348*, 154 months old) Japanese macaques. Representative images are presented. **a** HE staining of the subadult and adult Japanese macaque testes. Scale bars: 50 µm. **b** Immunofluorescence analyses of subadult and adult Japanese macaque testes with anti-DAZL, anti-VASA, and anti-calmegin antibodies. Nuclei were counterstained with DAPI. Scale bars: 50 µm

here due to limited availability of antibodies). This is needed to further elucidate the correspondence between the earlier classification and current molecular configuration for characterizing spermatogonial subtypes in the Japanese macaque.

The immunohistological atlas identified here broadens the molecular histological platform for future studies of postnatal development and seasonal breeding in the Japanese macaque. Indeed, comparative analyses between subadults and adults highlighted differences in their spermatogenesis status (Fig. 4), indicating that spermatogenic arrest in subadult testis is attributable to (1) restricted differentiation of spermatocytes and round spermatids, and (2) lack of synchronization of related processes in the seminiferous tubules. Previous studies have measured changes in endocrine factors associated with testicular development in the Japanese macaque (Hamada et al. 2005; Itoh et al. 2003; Matsubayashi et al. 1991; Sato et al. 2007). Combined analyses of molecular histology and endocrinology are thus likely to uncover associated spermatogenic characteristics in postnatal development and seasonal reproduction. Newer technologies such as small RNA profiling (Hirano et al. 2014; Yan et al. 2009) and mRNA-seq (Ramaswamy et al. 2017; Suzuki et al. 2019) will also greatly accelerate studies of the reproductive biology of the Japanese macaque, as well as open in vitro approaches to culture spermatogenic cells (Lin et al. 2016) or produce germ cells from stem cells (Imamura et al. 2014; Nakai et al. 2018) for this biomedically important species.

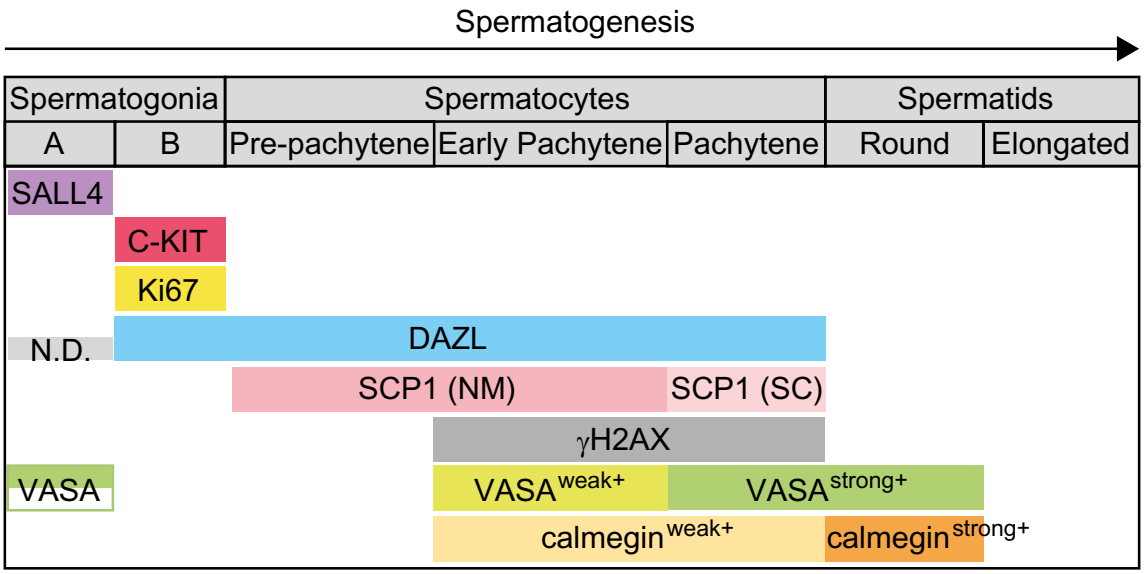

Fig. 5 Immunohistological dynamics throughout Japanese macaque spermatogenesis. Schematic representation of immunohistological characteristics in adult Japanese macaque spermatogenesis from spermatogonia to spermatids. Within the pink 'SCP1′ bar, NM and SC stand for nuclear marginal localization and synaptonemal complexes of SCP1 protein, respectively. The leftmost 'VASA' box is split green/white to indicate that A spermatogonia include $VASA^{+}$ and $VASA^{-}$ subtypes. $VASA^{+}$ A spermatogonia were $DAZL^{-}$, whereas it remains to be elucidated whether $VASA^{-}$ spermatogonia express DAZL. N.D., not determined

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Compliance with ethical standards

Conflict of interest The authors have no competing interests.

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