Research Article

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Stage-Specific Expression of CYP26B1 in the Adult Testis is Responsible for Pulsatile Retinoic Acid Signaling in Spermatogenesis

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Summary

Retinoic acid (RA) plays important roles in spermatogenesis process; however, mechanisms underlying pulsatile RA signaling in meiotic initiation has not been understood yet. We studied expression pattern of RA-degrading enzyme CYP26B1 during development of rhesus monkey testis using qPCR and IHC. In developing monkey testis, the CYP26B1 protein was detected in cytoplasm of undifferentiated spermatogonia. Expression level of CYP26B1 mRNA was down-regulated during testis development which is consistent with initiation of meiosis in adult testis. In adult testes, a heterogeneous pattern of CYP26B1 expression was observed along different stages of seminiferous epithelium, presumably indicates expression pattern of protein is stage specific. Highest level of CYP26B1 protein expression was coincided with onset of meiosis and observed in early meiotic spermatocytes within seminiferous epithelial stages X-XII. Whereas, lowest level of CYP26B1 expression was observed in stages VI-IX of seminiferous epithelium, where undifferentiated Type A spermatogonia divide and differentiate to Type B spermatogonia, meiosis initiates and spermiogenesis occurs. This observations led us to suggest CYP26B1 might be responsible for pulsatile RA signaling in spermatogenesis. Findings presumably support that elevated amount of RA in undifferentiated Type A spermatogonia during stages VI-IX of the seminiferous epithelium of adult monkey testis may be responsible for differentiation of spermatogonia and their meiotic entry.

Introduction

Spermatogenesis is a continuous process of spermatozoa production which classically has been divided into three distinct phases including spermatogonia proliferation by mitosis, two reduction divisions by meiosis that yield haploid spermatids, and differentiation of haploid spermatids into mature spermatozoa (spermiogenesis) [1-3].

As the same as rodent testis, twelve different morphological stages (I-XII) have been identified in the rhesus monkey testis, which associated with 14 developmental steps (1-14) of the spermatid [4,5]. The duration of spermatogenesis in the rhesus monkey from spermatogonia to elongated spermatid is approximately 42 days [5]. The duration of each cycle of the seminiferous epithelium covering the 12 stages is abou10.5 days [5-7].

In the adult rhesus monkey, undifferentiated Type A pale (Ap) spermatogonia during Stages VII-IX divide to double their number; half of the new generation of Ap then divide to give rise to differentiating B1 spermatogonia (differentiation), and the other half remain as Ap (renewal) [8-10].

The spermatogenesis process starts at puberty and maintains by proliferation and differentiating division of spermatogonial germ cells that reside at the basement membrane of seminiferous epithelium [1,2,11]. In mammals, the spermatogenesis onset and spermatogonia commitment to differentiate depends on the effect of different factors on the testis such as gonadotropins and vitamin A; however, in the absence of gonadotropins and vitamin A deficiency, testis display a halt at spermatogonia differentiation and only undifferentiated germ cells and Sertoli cells are present in the testis [12-15].

A large number of studies have revealed which retinoic acid (RA), the major physiologically active form of vitamin A, plays vital roles in different developmental processes such as spermatogenesis [16,17]. The RA signaling occurs in a stage-specific manner during the spermatogenic cycle in the rodents [18]. It has been revealed that RA signaling plays crucial roles during the two stages of seminiferous epithelium cycles VII and VIII, where spermatogonial differentiation, the initiation of meiosis, and spermiogenesis occurs, which indicates activation of RA signaling is only necessary during these stages [15,18-21].

The metabolism of vitamin A, also known as retinol (ROL), to retinoic acid (RA) is a two-step enzymatic process in the target cells [22]. In the first step, retinol is converted into retinaldehyde (RAL) through the reversible oxidation that involves alcohol dehydrogenase or retinol dehydrogenase (RDH) enzymes [22]. In the next step, retinaldehyde

is converted into retinoic acid through irreversible oxidation that is catalyzed by retinaldehyde dehydrogenase (RALDH) enzymes. RA can be either bind to the retinoic acid receptors (RARs), and thereby bind to retinoic acid response elements (RAREs) in the genome, through dimerization with retinoid X receptors (RXRs), and induce the expression of specific genes or degraded via the cytochrome P450 family 26 (CYP26) enzymes [21]. Degradation of RA is a crucial step for protection of cells from excessive stimulation of RA. Three cytochrome P450 hydroxylases, CYP26A1, CYP26B1 and CYP26C1 are responsible for hydroxylation and thereby inactivation of RA [23-24].

Studies of mutant phenotype have shown that XY Cyp26b1 (cytochrome P450, family 26, subfamily B, polypeptide1) null germ cells enter meiosis and express meiotic markers, and it is essential for postnatal survival and germ cell development. In the fetal testis, CYP26B1 degrades RA to suppress meiosis [23,25-27]. Previous study revealed that Cyp26b1 transcripts and CYP26b1 protein did not change significantly during mouse postnatal testis development [28]. In the developing and adult testis, CYP26b1 protein located in peritubular myoid cells at the outer layer of the seminiferous tubule, in all stages [28,29]. Expression of CYP26B1 in Sertoli cells acts as a masculinizing factor to arrest male germ cells in the G0 phase of the cell cycle and prevents them from entering meiosis, and thereby maintain the undifferentiated state of male germ cells during embryonic development [30].

Current knowledge about control of germ cell development by the RA signaling molecules relies on more recent studies among rodents, while studies among higher mammals such as humans and non-humans primates remains poorly understood. In the other hand, the actual level of retinoic acid proteins at different stages of seminiferous epithelium development in the testis is unknown due to a lack of relevant antibodies. The question is if the expression of RA and its signaling occurs only by stage-specific manner, how this phenomenon happens? We proposed the expression of RA-degrading enzyme CYP26B1 is change during different stages of testis development and seminiferous epithelium, which reflect the level of RA in the different seminiferous tubules. Therefore, we were interested in examine the expression of CYP26B1 in both mRNA and protein levels as well as its protein localization during development of monkey testis, which provide evidences for regulatory mechanisms of RA signaling in the cycle of seminiferous epithelium.

Material and Methods

Animals and Testicular Tissues

Six juvenile (14-17 months of age) and adult (age 6.1 to 12 years) male rhesus monkeys (Macaca mulatta) with normal weight and testicular volume were included in this study. The animals were maintained in the Primate Research Facility in accordance with NIH Guidelines for the Use and Care of Experimental Animals approved by the Institutional Animal Committee on Use and Care. Testicular tissues from each monkey were obtained by castration through a surgical process after anesthesia. Testes were cut into several portions and some of them were fixed in 4% paraformaldehyde (PFA) and Bouin's fluid for histological analysis. Some tissues were frozen in liquid N_2 and stored at -80°C for RNA extraction.

RNA isolation and cDNA preparation

RNA was obtained from testicular tissues using RNA isolation method developed by Chomczynski and Sacchi [31]. After digestion with ribonuclease-free deoxyribonuclease, the RNA was subjected to reverse transcription using random hexamers. For reverse transcription, 250 ng of RNA was incubated with 50 µl of reaction mix containing 25 mM MgCl2, 25 mM deoxynucleotide triphosphates (Promega, Madison, WI), 10x PCR II Buffer (Applied Biosystems), 20 U RNasin ribonuclease inhibitor (Promega, Madison, WI), 2.25 µm random hexamers (Integrated DNA Technologies, Coralville, IA), 125 U SuperScript reverse transcriptase II (Invitrogen), and nuclease-free water (Ambion, Austin, TX). Parallel reactions were performed without reverse transcriptase to control for the presence of contaminant DNA. The samples were incubated in PCR thermocycler at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min followed by 4 °C for 5 min.

Gene expression analysis by quantitative real-time PCR (qPCR)

Analysis of gene expression was performed by qPCR amplifications in a 96-well plate in the ABI Prism 7900HT sequence detection system version 2.3 (Applied Biosystems, Foster City, CA) in a total volume of 20 µl, which included 2 µl of cDNA, 10 µl of Perfecta SYBR Green FastMix ROX (Quanta Biosciences, Inc., Gaithersburg, MD), and 600 µm of each primer. Primers were positioned in different exons of gene to avoid false positive because of probable DNA contamination during RNA extraction and independently validated for use in the ΔΔCt method of gene expression analysis. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an endogenous control. The qPCR analysis initiated with melting of cDNA at 95 °C for 15 min, followed by 40 amplification cycles (95 °C for 15 sec and 60°C for 1 min). A dissociation curve was performed immediately after amplification to ensure there was only one (gene specific) amplification peak. Cycle threshold (Ct) values were recorded and analyzed via the ΔΔCt method [32]. The means (±sd) of three individual experiments were determined for each treatment group for each gene of interest. Primers used for each gene are listed in **Table 1**.

Table 1: Primers used for qPCR

Gene	Accession no.	Sequence (5′–3′)	Region amplified	Tm	product Size
CYP26B1	XM_001103025	CTTCTCCCTGCCTGTTGACC TGCTCCTTGCTGCTCTCAAT	460 627	60.32 59.67	168
GAPDH	NM_001195426	CCATCTTCCAGGAGCGAGATC GCTCCCCCCTGCAAATG	284 407	60.00 57.91	124

Immunofluorsence Staining

Testes fragments were fixed in 4% PFA and then embedded in paraffin. Paraffin-embedded tissues were cut at 5 µm thickness and used for immunofluorsence staining. Testis sections (5 µm thick) were deparaffinized in xylenes and rehydrated by a series of descending concentrations of ethyl alcohol and washed in 1X PBS (0.1 M, pH 7.3). The sections were then subjected to antigen retrieval for 1 hour at 97.5°C in ethylenediaminetetraacetic

acid (EDTA) buffer (1 mM, pH 8.0) with 0.05% Tween-20, and allowed to cool at room temperature for 30 minute and washed in PBST buffer (PBS with 0.1% Tween-20, pH 7.3). The sections then incubated in blocking buffer solution (PBS with 10% normal donkey serum, 3% BSA and 1% Triton X-100) for 30 minute at room temperature in a humid dark box. Then sections were incubated in the respective primary antibody **(Table 2)** overnight at 4°C in a humid dark box. Sections were washed in 1x PBST. Sections were incubated in a mixture of secondary antibody (1:200) for 45 min at room temperature in a dark humidified box. Sections were washed in 1x PBST, and then cleared in xylenes and cover slipped with Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL, USA). For controls, testis sections from an adult monkey were processed for the immunoflourescence procedures described above, but with selective substitution of the primary antibody CYP26B1 (Figure 2).

Table 2: Antibodies used for immunofluorescence staining

1st Antibody	Species	Company	Cat. #	Concentration (Dilution)	2nd Antibody
CYP26B1	rabbit polyclonal anti-human	LifeSpan Bioscience, inc. USA	LS-B4833	1 mg/ml (1:100)	Alexa Fluor 488-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.)
CYP26B1	rabbit polyclonal anti-human	Sigma-Aldrich Inc., MO, USA	B37589	0.21 mg/ml (1:200)	Alexa Fluor 488 conjugated AffiniPure Donkey anti Rabbit (Jackson ImmunoResearch Laboratories, Inc.)

Immunohistochemical localization analysis

A portion of testes fragments were fixed in Bouin's fluid and then embedded in paraffin. The Bouin's fixed paraffin-embedded tissues were cut at 5 µm thickness and were analyzed immunohistochemicaly for localization of CYP26B1. For this purpose, two sections from a testis of each monkey were deparaffinized in xylene and rehydrated with decreasing concentrations of ethanols, followed by washing in PBS. The sections subjected to antigen retrieval in EDTA buffer (1 mM EDTA, pH 8.0, 0.05% Tween-20) for 60 min using a pre-heated water bath set to 97.5°C. The sections were allowed to cool at room temperature before continuing with the immunostaining procedure. Endogenous peroxidases were inactivated using hydrogen peroxide solution (Sigma-Aldrich; St Louis, MO, USA), and then blocking was carried out using 5% normal goat serum (in 1x PBS, Triton X-100, 3% BSA) for 45 min at room temperature. Sections were then incubated overnight at 4°C with CYP26B1 polyclonal rabbit anti-human (Sigma-Aldrich; St Louis, MO, USA) diluted 1:200 in 50 mM PBS containing 0.05% Triton X-100 and 5% normal donkey serum. After several rinses with PBS, sections were incubated at room temperature with biotinylated goat anti-rabbit antiserum (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:200 in PBS/Triton and 5% normal goat serum for 1 h. After rinsing, sections were placed in a solution of an avidin–horseradish peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at room temperature. After washing in PBS, horseradish peroxidase was visualized with 3,3′-diaminobenzidine (SigmaFast DAB/Cobalt, Sigma Chemical Co.). The sections were counterstained with periodic acid schiff (PAS)-hematoxylin, dehydrated, cover slipped with Fluoromaunt and examined with a Nikon 90i microscope (Nikon; Tokyo, Japan). For negative controls, the primary antibody was replaced with 5% normal goat serum. Dark brown precipitate indicated the presence of CYP26B1. Immunostaining of sections was repeated at least three times.

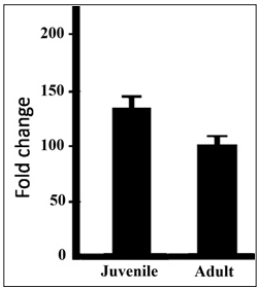
Data Acquisition and Analysis

The scheme described by Clermont and Antar was used for staging of the seminiferous epithelium but was significantly aided by the stage diagram published by Dreef et al. [7,8]. For this purpose, after confirming reproducibility of results using the immunohistochemistry procedure, five random sections and five consecutive sections from each testis were stained immunohistochemicaly. After ensuring that all sections were treated in an identical fashion and subjected to identical times in all steps of immunohistochemistry staining, staining pattern was evaluated and scored at the completion of the immunohistochemistry staining procedure. To reduce probable edge artifacts, which interfere with the scoring of the immunohistochemistry staining, tubules at the middle of the sections were investigated. At least twenty seminiferous tubules were counted from each testicular section. Because it was difficult to typically distinguish between stages I-V and between stages X-XII, data from these stages were pooled. In addition, results from stage VI and IX were pooled with those from stages VII-VIII. Images were captured using a Qimaging scientific research camera (Qimaging, Inc.; Surrey, Canada) in a Nikon 90i microscope (Nikon; Tokyo, Japan).

Results

Analysis of CYP26B1 expression at mRNA level during monkey testis development

The expression level of CYP26B1 mRNA at different stages of monkey testes was determined by qPCR analysis (Fig. 1). The expression of CYP26B1 mRNA began to decrease during monkey testis development. As shown in **(Figure 1)**, the relative amount of CYP26B1 mRNA expression slightly down-regulated from juvenile to adulthood, but it's not significant (Figure 1).



Analysis of CYP26B1 expression at protein level during monkey testis development

The expression of CYP26B1 at protein level in developing and adult monkey testis was determined in 4% PFA sections by immunofluorescence staining **(Figure 2)**. Using indirect immunofluorescence, CYP26B1 protein was detected during testis development from juvenile (14-17 months of age) until adulthood (> 6 years of age). As shown in Figure 2, CYP26B1 was only expressed in the cytoplasm of spermatogonia in the all seminiferous tubules in the developing testis, whereas all non-germ cells including Sertoli cells, interstitial cell, and peritubular mioid cells were CYP26B1 negative (Figure 2A). Interestingly; in the adult testis, the CYP26B1 signal was detected at cytoplasm of pre-meiotic germ cells and some interstitial cells; whereas Sertoli cells, spermatocytes and elongated spermatids were CYP26B1 negative (Figure 2B). A rather heterogeneous pattern of the CYP26B1 protein expression was observed in the different stages of seminiferous epithelium cycles, which indicating the expression of CYP26B1 is stage specific (Fig. 2B). Robust fluorescent signals of CYP26B1 were observed in meiotic germ cells (Fig. 2B). Relative to the adult testis, the amount of CYP26B1 protein signal was higher in the germ cells of the juvenile monkey testis, which was consistent with their relative mRNA level. Negative control using normal donkey IgG instead of anti-CYP26B1 antibodies did not show any positive signals.

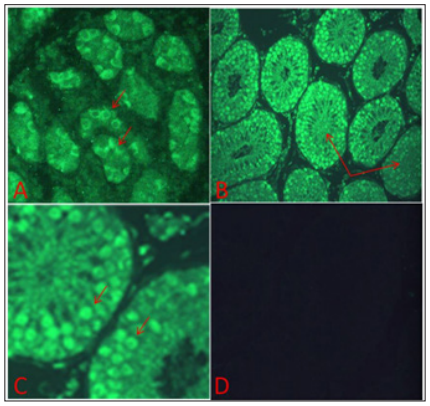


Figure 2: Expression of CYP26B1 in developing and adult monkey testis was determined using immunofluorcence staining. (A–D) Immunofluorcence localization in monkey testicular tissue, (A) showing pronounced cytoplasmic localization of the CYP26B1 in spermatogonia lining the basement membrane of seminiferous epithelium of juvenile testis (arrows). (B) Localization of CYP26B1 in pre-meiotic and meiotic germ cell of adult testis (arrows). The staining was heterogeneous throughout the tubule, indicating stage specificity. (C) Higher magnification of (B). (D) Negative control. Higher level of CYP26B1 was detected in the juvenile, similar to results obtained by qPCR.

Localization of CYP26B1 protein in the seminiferous epithelium of adult monkey testis

The Bouin's fixed sections were analyzed using immunohistochemical staining followed by hematoxylin for better understanding of CYP26B1 localization in different stages of seminiferous epithelium. As shown in Figure 3A and 3B, the staining of CYP26B1 was heterogeneous during different stages of seminiferous tubules, which is consistent with the staining result of immunofluorescence. Immunohistochemical signals of CYP26B1 were observed in early meiotic germ cells including preleptotene, zygotene and pachetene spermatocytes at some seminiferous epithelial stages in the adult monkey testis, whereas undifferentiated Type A spermatogonia and post meiotic germ cells including round and elongated spermatids were negative. Interestingly, robust immunohistochemical signals of CYP26B1 were observed in seminiferous epithelial stage of X-XII, and moderate CYP26B1 signal was observed in epithelial stage of I–V, whereas CYP26B1 signal in seminiferous epithelial stages VI-IX was almost negative **(Figure 3)**.

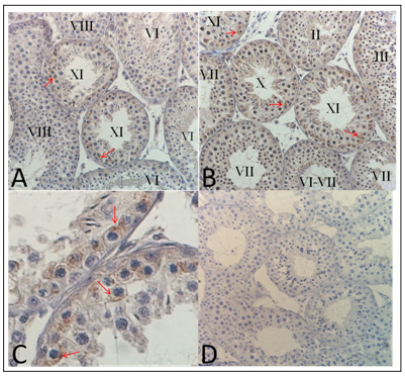


Figure 3: Localization and stage-specific expression of CYP26B1 in the adult monkey testis. (A-D) Localization of CYP26B1 in adult monkey testicular tissue by immunohistochemical staining, (A and B) showing marked cytoplasmic localization of the CYP26B1 enzyme in the cytoplasm of pre-meiotic and meiotic germ cells (arrows). The staining was heterogeneous throughout the tubule, indicating stage specificity. (C) Higher magnification of (A). (D) Negative controls using normal goat IgG instead of anti-CYP26B1 antibody did not show any positive signals.

Differential Expression of CYP26B1 protein in Stage Clusters of Seminiferous Tubules

As the expression of CYP26B1 protein in the adult testes was not uniform among spermatocytes, perhaps indicating the asynchronous beginning of spermatogenesis, we sere interest to investigate whether the expression of CYP26B1 protein was present in all stages of the seminiferous tubules or if it was stage specific. Three stage clusters of seminiferous tubules were manually dissected from adult mouse testes, including stages I-V (moderate spot), stages VI-IX (weak spot), and stages X-XII (strong spot) [33]. As shown in **Figure 4**, the level of CYP26B1 protein was significantly higher in stage X-XII tubules than in the other stages tested which indicating the expression of this protein is stage specific. Moreover, the expression pattern of CYP26B1 indirectly reflects that the RA levels is high during the stages of X-XII seminiferous tubules of the monkey testis.

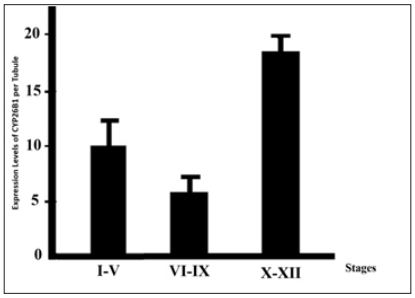


Figure 4: The CYP26B1 protein expression in stage clusters of adult seminiferous tubules. Values represent levels of CYP26B1 protein expression in three stage clusters of adult seminiferous tubules ±SEM.

Discussion

Recent studies indicated that the expression of the RA metabolizing enzyme Cyp26b1 in the premature testis shields germ cells from the meiosis-inducing action of RA. Studies from XY CYP26B1-null mice indicate that RA accumulation is vital for meiotic initiation. Studies of germ cell deficient in CYP26B1 indicated that RA is responsible for meiotic initiation. A significance increase in RA levels was shown in the embryonic testis of Cyp26b1-null mice [26]. The inhibition of CYP26B1 with ketoconazole in the fetal testis has shown an up-regulation in Stra8, Scp3, and Dmc1 levels, and the germ cells shown morphological characteristics of meiotic germ cells [17,25]. An increase in the expression of Stra8 and Sycp3 as well as the presence of pachytene-like spermatocytes was observed by embryonic day 16.5. However meiotic XY germ cells are removed by apoptosis during late fetal life [25,26]. The inhibition of Cyp26b1 Ketoconazole in Raldh2 (-/-) testis allows induction of Stra8 in RA independent manner, and demonstrated that Cyp26b1 prevents the meiosis entry by metabolizing a substrate other than RA that controls Stra8 expression [34]. In the treated larval testes from frogs with exogenous RA or CYP26 inhibitors, leptotene spermatocytes were observed [35]. Whereas leptotene spermatocytes were absent when larval frog testes were cultured with either an RALDH inhibitor or an RAR antagonist [35]. All these studies indicate inhibition RA signaling degrading enzyme CYP26B1 is important for meiotic initiation and progression.

Recent studies have revealed that the RA signaling occurs in a stage-specific manner during the spermatogenic cycle in the rodents, and it plays vital roles during the two stages of seminiferous epithelium cycles VII and VIII, where spermatogonial differentiation, the initiation of meiosis, and spermiogenesis occurs, indicating activation of RA signaling is only necessary during these two stages [15,18-21]. The main question is if the expression of RA and its signaling occurs only by stage-specific manner, how this phenomenon happens? It has been supposed that the expression pattern of RA-degrading enzymes in the adult testis might be in a stage-specific manner [18]. To this end, we were interested in evaluating the expression of the RA-degrading enzyme CYP26B1 during development of monkey testis at both mRNA and protein levels.

Our qPCR results showed that the expression of CYP26B1 was decreased during development of monkey testis, although the effect was not significant. During the post-natal testicular development, the RA metabolizing enzymes CYP26b1 and ALDH1 genes were turned down and up respectively [36]. However, down-regulation of CYP26B1 from juvenile to adulthood in our result is consistent with initiation of meiosis and spermatogenesis process in the adult testis.

A comprehensive study on the mouse testis conducted by Vernet et al.has revealed that cells within the seminiferous epithelium are responsible for synthesizing and controlling of RA levels [29]. As reported by Bowles et al. and McLean et al. CYP26B1 plays as the meiosis inhibitor in the embryonic testis to prevent RA production and, as a result, male embryonic germ cells undergo G0/G1 mitotic cell cycle arrest, and meiosis does not begin until puberty. Our results shown that the pattern of RA degrading molecule within the seminiferous tubule is different from the juvenile to the adult monkey testis to maintaining spermatogenesis. The localization of CYP26B1 expression pattern demonstrated that it is only expressed in the undifferentiated spermatogonia in the all seminiferous tubules in the developing testis, which is consistent with inhibition of RA signaling and meiotic entry in the developing monkey testis. Wherease; the localization of CYP26B1 protein was shifted from undifferentiated spermatogonia to the meiotic germ cells in the adult testis in a stage specific manner. The CYP26B1 signal was only detected at pre-meiotic and early meiotic germ cells including leptotene, zygotene and pachetene spermatocytes in the seminiferous tubules of the adult monkey testis, while undifferentiated spermatogonia and post-meiotic germ cells including round and elongated spermatids were CYP26B1 negative. The stage specific localization of CYP26B1 in early meiotic spermatocytes within seminiferous epithelium might be responsible for pulsatile RA signaling and meiotic entry in the adult testis. The higher expression of CYP26B1 in the seminiferous epithelial stage of X-XII reflects the lower level of RA in these stages; whereas, weak CYP26B1 signal in seminiferous epithelial stages VI–IX reflects the highest level of RA in this stages of seminiferous tubules in the monkey testis where differentiation of spermatogonia, initiation of meiosis and spermiogenesis occurs.

In summary

As previously described, the elevated amount of CYP26B1 expression in the undifferentiated spermatogonia at juvenile testis degrade synthesized RA in spermatogonia and thereby prohibit RA signaling and meiotic entry in the developing testis. Whereas, in the adult testis, the excessive amount of RA in the seminiferous epithelium (stages VI-IX) due to down-regulation of CYP26B1 might be responsible for differentiation of undifferentiated spermatogonia and their meiotic entry. Moreover, up-regulation of CYP26B1 in specific stages (X-XII) within the somniferous epithelium can be responsible for degradation of RA activity and protecting germ cells from excessive amount of RA in the adult testis and thereby might be consider for generating pulsatile RA signaling in spermatogenesis. However, the molecular mechanism controlling the stage specific expression of CYP26B1 remains to be clarified.

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Author's contributions

SN: doing experiments, study conception and design, acquisition of data, analysis, interpretation of data; preparation of the manuscript, revision and approval of the manuscript. BR: revision and approval of the manuscript, preparation of the manuscript.

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