

·Original Article·

Expression of the retinoic acid-metabolizing enzymes RALDH2 and CYP26b1 during mouse postnatal testis development

Jing-Wen Wu^{1,2}, Ru-Yao Wang^{1,2}, Qiang-Su Guo^{1,2}, Chen Xu^{1,2}

¹Department of Histology and Embryology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

²Shanghai Key Laboratory for Reproductive Medicine, Shanghai 200025, China

Abstract

Aim: To study the expression pattern of the retinoic acid metabolizing enzymes RALDH2 and CYP26b1 during mouse postnatal testis development at both mRNA and protein levels. **Methods:** Real-time polymerase chain reaction and Western blot analysis were performed to determine the relative quantity of RALDH2 and CYP26b1 at both mRNA and protein levels at postnatal day 1, 5, 10, 20, and in adult mice (70 days testes). Testicular localization of RALDH2 and CYP26b1 during mouse postnatal development was examined using immunohistochemistry assay. **Results:** *Aldh1a2* transcripts and its protein RALDH2 began to increase at postnatal day 10, and remained at a high level through postnatal day 20 to adulthood. *Cyp26b1* transcripts and CYP26b1 protein did not change significantly during mouse postnatal testis development. RALDH2 was undetectable in the postnatal 1, 5 and 10 day testes using immunohistochemistry assay. At postnatal day 20 it was detected in pachytene spermatocytes. Robust expression of RALDH2 was restricted in round spermatids in the adult mouse testis. In the developing and adult testis, CYP26b1 protein was confined to the peritubular myoepithelial cells. **Conclusion:** Our results indicate that following birth, the level of retinoic acid in the seminiferous tubules might begin to increase at postnatal day 10, and maintain a high level through postnatal day 20 to adulthood. (*Asian J Androl* 2008 Jul; 10: 569–576)

Keywords: RALDH2; CYP26b1; retinoic acid; spermatogenesis; testis

1 Introduction

Spermatogenesis is a highly regulated process of differentiation that can be subdivided into three main phases: spermatogonial proliferation, meiosis of spermatocytes and spermiogenesis of haploid spermatids. This process requires a complex assortment of hormones and cytokines

[1, 2]. Among these signals, many studies have demonstrated that retinoic acid (RA) could play an indispensable role in spermatogenesis by promoting spermatogonia differentiation, adhesion of germ cells to Sertoli cells, and the release of mature spermatids into the lumen of seminiferous tubules [3, 4]. In order to appropriately stimulate the retinoid signaling pathway during spermatogenesis, RA synthesis and degradation must be spatiotemporally regulated.

Retinoic acid is predominantly produced from dietary vitamin A (retinol, ROL) through a two-step metabolic pathway [5]. The first step, reversible oxidation of ROL into retinaldehyde, involves either alcohol dehydrogenases, or microsomal retinol dehydrogenases, which are mem-

Correspondence to: Dr Chen Xu, Department of Histology and Embryology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China.

Tel: +86-21-6384-6590 ext. 776435 Fax: +86-21-6466-3160

E-mail: chenx@shsmu.edu.cn

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bers of the short-chain dehydrogenase/reductase family (SDR). The second step, irreversible oxidation of retinaldehyde into RA, is catalyzed by four retinaldehyde dehydrogenases (RALDH1, 2, 3, 4, encoded by the *Aldh1a1*, *Aldh1a2*, *Aldh1a3* and *Aldh8a1* genes, respectively) [5, 6], of which *Aldh1a1* and *Aldh1a2* are expressed in the rodent testis [7, 9]. Using *in situ* hybridization, *Aldh1a1* transcripts were detected in the Leydig cells, whereas the transcripts of *Aldh1a2* were only detected in the germ cells in adult mouse testis, suggesting that RALDH2 appears to be responsible for essentially all RA synthesis within the seminiferous epithelium [10].

Aside from synthesis, degradation of RA is also an important balancing mechanism that protects cells from excessive RA stimulation [11]. It is catalyzed by at least three cytochrome P450 hydroxylases (CYP26A1, CYP26B1 and CYP26C1), which repeatedly hydroxylate RA and its metabolites into increasingly water-soluble products that are less active and readily excretable [12]. Recently, it was reported that the mRNAs of all three RA-degrading enzymes are expressed in the peritubular myoepithelial cells [10]. Because of this, the excessive RA made inside the seminiferous tubules can be degraded to permit a suitable RA stimulation and any RA made outside of the seminiferous tubules is, therefore, unlikely to reach the Sertoli cells or the germ cells.

Considering the distribution of RA-synthesizing and RA-degrading enzymes in the mouse testis studied previously, we may infer that germ cells in the seminiferous tubules have to synthesize RA by themselves through RALDH2 and the excessive RA can be degraded through CYP26b1. However, all these findings are based on localizing the transcripts of the RA metabolizing enzymes due to a lack of relevant antibodies to detect the actual proteins themselves. In addition, the level of RA in the seminiferous tubules at different stages of mouse postnatal development remains unknown up to now. We hypothesized that the balance of RALDH2 and CYP26b1 might reflect the level of RA in the seminiferous tubules. Therefore, the objective of the present study was to determine the relative quantity of RALDH2 and CYP26b1 at both mRNA and protein levels as well as their protein localization during mouse postnatal testis development.

2 Materials and methods

2.1 Animals

Male BALB/c mice and male white New Zealand rab-

bbits (approximately 6 months old, body weight approximately 2.5 kg) were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained at the Laboratory Animal Center of Shanghai Jiaotong University School of Medicine under the animal welfare guidelines of our school. Mice were divided into five groups ($n = 3$) according to their postnatal age (1, 5, 10, 20 and 70 days; the day of birth was considered day 0) and testes were removed immediately after killing the animals by cervical dislocation. The testes were either placed in RNAlater (Qiagen, Hilden, Germany) until being used for real-time polymerase chain reaction (PCR), or frozen immediately in liquid nitrogen and then stored at -80°C until further processing for indirect immunofluorescence assay.

2.2 Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was performed for analysis of the *Aldh1a2* and *Cyp26b1* mRNA expression during postnatal mouse testis development. Total RNA was extracted from postnatal day 1, 5, 10, 20 and 70 mouse testes using RNeasy Mini according to the manufacturer's protocol (Qiagen) and cDNA was generated from 1 μg of total RNA using the Promega reverse transcription system (Promega Corp., Madison, WI, USA). The reaction was incubated at 25°C for 10 min, at 42°C for 1 h, and finally at 95°C for 5 min. For qPCR, 2 μL cDNA was used in a 18 μL SYBR premix reagent (Takara, Dalian, China). Amplification was then performed in duplicate using the following primer sets: 5'-CAT CCA CCG CAA CAA GC-3' (sense) and 5'-CCA TTC GGA AGG TAA GTC G-3' (antisense) for *Cyp26b1*; 5'-AAT CCC TAA ATG GCG GTA-3' (sense) and 5'-ATG GGC TCG TGT CTT GTG-3' (antisense) for *Aldh1a2*; and 5'-CAG CCT TCC TTC TTG GG-3' (sense) and 5'-GGC ATA GAG GTC TTT ACG G-3' (antisense) for β -actin. PCR was carried out in an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) with denaturation at 95°C for 15 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 31 s. The relative mRNA levels of *Aldh1a2* and *Cyp26b1* in each sample were calculated using the comparative C_T method ($\Delta\Delta C_T$). Briefly, β -actin was used as an endogenous gene for normalization. Its average C_T value was subtracted from that of the *Aldh1a2* or *Cyp26b1* to obtain ΔC_T . Calculation of $\Delta\Delta C_T$ involves subtracting the ΔC_T values from the ΔC_T calibrator value

(ΔC_T of 1 day testis). The relative quantitative value was expressed as fold difference that calculated by $2^{-\Delta\Delta C_T}$, representing the amount of *Aldh1a2* or *Cyp26b1* expression, normalized to the endogenous gene (β -actin) and relative to the calibrator (1 day testis).

2.3 Production of polyclonal rabbit anti-RALDH2 and anti-CYP26b1 antibodies

Healthy white New Zealand rabbits were immunized with polyHis-tagged recombinant fragments of protein RALDH2 (approximately 27 kDa) and CYP26b1 (approximately 22 kDa) generating from the prokaryotic expression system, as described in Cheng *et al.* [13] and Hu *et al.* [14]. At day 35 after immunization, anti-sera were collected and purified using the montage antibody purification kit (Millipore, Billerica, MA, USA).

2.4 Protein extraction and bicinchoninic acid protein assay

Different aged of the mouse testes' whole proteins were extracted using T-per tissue protein extraction reagent (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The concentrations of the proteins were determined using a bicinchoninic acid protein assay kit (Pierce).

2.5 Electrophoresis and Western blot analysis

Recombinant proteins and proteins extracted from different ages of the mouse testes were thawed in prewarmed $2 \times$ SDS-PAGE sample loading buffer (80 mmol/L Tris-HCl [pH = 6.8], 20% glycerol, 4% SDS, 4% β -mercaptoethanol, 0.04% bromophenol blue), vortexed and then denatured at 95°C for 10 min and placed on ice for 5 min. Proteins were loaded in each lane (5 μ g per lane for recombinant proteins and normal rabbit IgG; 30 μ g per lane for mouse testis whole protein extracts) and separated by SDS-PAGE. Resolving gels were cast using 12% acrylamide; stacking gels contained 5% acrylamide. Gels were equilibrated in TBS with Tween (TBST) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) by Semi-Dry Electrophoretic Transfer (Bio-Rad Laboratories, Hercules, CA, USA). Blots were blocked in 5% nonfat milk in TBST at room temperature for 1 h and incubated with anti-RALDH2 antibody (diluted 1:4 000), anti-CYP26b1 antibody (diluted 1:4 000), anti-His monoclonal antibody (diluted 1:2 000) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody diluted

1: 10 000 in TBST plus 5% nonfat milk overnight at 4°C with agitation. After complete washing in TBST, blots were incubated with anti-rabbit horseradish peroxidase conjugated IgG (diluted 1:10 000) or anti-mouse horseradish peroxidase conjugated IgG (diluted 1:4 000) at room temperature for 1 h, washed in TBST, and developed with ECL Plus reagents (Millipore).

2.6 Indirect immunohistochemistry assay

Testes from -80°C for frozen sections were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and cut at 10 μ m thickness. Sections were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min, permeabilized with acetone for another 15 min, and blocked for 1 h with block solution (10% normal goat serum in PBS). The anti-RALDH2 and anti-CYP26b1 antibodies were diluted to 1:200 in block solution and used for incubation overnight at 4°C. For the negative control sections, the anti-RALDH2 and anti-CYP26b1 were replaced with normal rabbit IgG. After washing in PBS, sections were incubated with FITC-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) diluted to 1:200 for 2 h at room temperature. Flowing wash coverslips were mounted on the glass slides with mounting medium containing DAPI (Vector, Burlingame, CA, USA). Microscopic images were obtained on a confocal microscope (Zeiss, Jena, Germany). This experiment was repeated twice. After taking photographs, the immunofluorescence stained sections were restained with hematoxylin/eosin and photos were taken again for cell discrimination.

3 Results

3.1 Relative amount of *Aldh1a2* and *Cyp26b1* mRNA during mouse postnatal testis development

Both *Aldh1a2* and *Cyp26b1* mRNA were detected during postnatal testis development from postnatal day 1 to adulthood (70 days). As shown in Figure 1, relative to postnatal day 1 testis, the expression level of *Aldh1a2* mRNA was decreased at 5 d (0.31-fold relative to 1 day), and then it increased slightly at 10 days (1.19-fold). From postnatal day 20 to adulthood, the amount of *Aldh1a2* mRNA increased distinctly (3.39-fold in 20 days and 4.72-fold in adulthood relative to 1 day testis). Relative *Cyp26b1* mRNA expression at different stages of the postnatal testes did not change significantly (Figure 1).

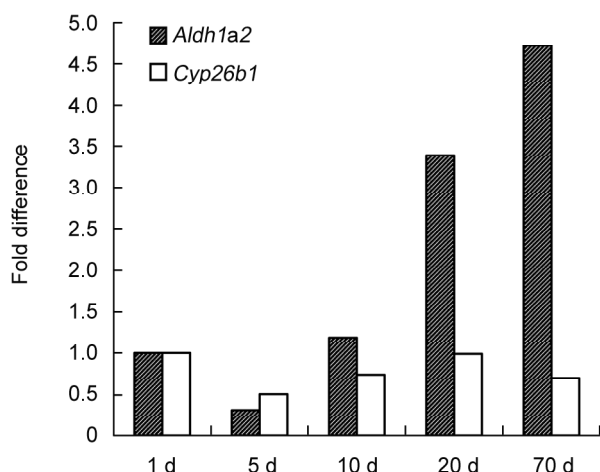


Figure1. Relative quantity of *Aldh1a2* mRNA (black column) and *Cyp26b1* mRNA (white column) at different ages of mouse testes using the comparative C_T method.

At 5, 10, 20, and 70 days, the amount of *Cyp26b1* mRNA was 0.50-, 0.74-, 0.99- and 0.70-fold relative to that of day 1, respectively.

3.2 Specificity of polyclonal anti-RALDH2 and anti-CYP26b1 antibodies

Recombinant RALDH2 and CYP26b1 protein fragments and adult mouse testis protein extracts were separated on a 12% SDS-PAGE gel and transferred to PVDF membrane for immunoblotting using anti-RALDH2, anti-CYP26b1 or anti-polyHis-mAb to characterize the specificity of the antibodies. The recombinant RALDH2 protein fragment (27 kDa) and its full length protein (54.5 kDa) in mouse testis were both detected using anti-RALDH2 antibody (Figure 2A). Similarly, the 22 kDa CYP26b1 recombinant fragment and its 57 kDa full length CYP26b1 protein both reacted with anti-CYP26b1 antibody (Figure 2B). The His-tagged recombinant proteins were also detected using anti-polyHis. Probing with normal rabbit IgG did not show any such bands. These results demonstrated the specificity of the anti-RALDH2 and anti-CYP26b1 antibodies.

3.3 Quantification of RALDH2 and CYP26b1 protein during mouse postnatal testis development

The protein levels of RALDH2 and CYP26b1 at different ages of mouse testes were determined by Western blot (Figure 3). GAPDH was used as an endogenous protein for normalization. RALDH2 and CYP26b1 were

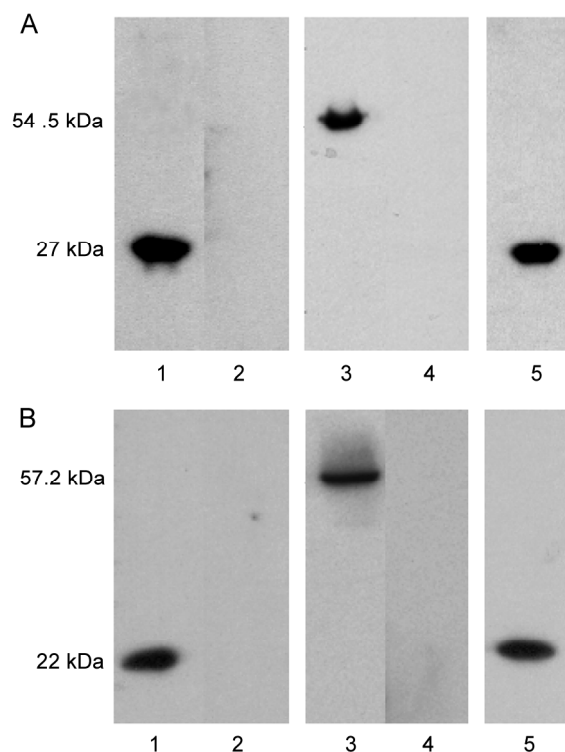


Figure 2. The specificity of the anti-RALDH2 antibody (A) and anti-CYP26b1 antibody (B) was determined by Western blot. Purified recombinant RALDH2 protein (Lane 1 in A) and full length RALDH2 protein in adult mouse testis whole protein extracts (Lane 3 in A) both reacted with anti-RALDH2 antibody; and anti-CYP26b1 antibody also detect recombinant CYP26b1 protein (Lane 1 in B) and full length CYP26b1 protein in adult mouse testis whole protein extracts (Lane 3 in B); while no reaction was observed with the addition of normal rabbit IgG (Lane 2 and Lane 4 in A and B, respectively). The His-tagged recombinant proteins were also detected using anti-polyHis monoclonal antibody (Lane 5 in A and B, respectively).

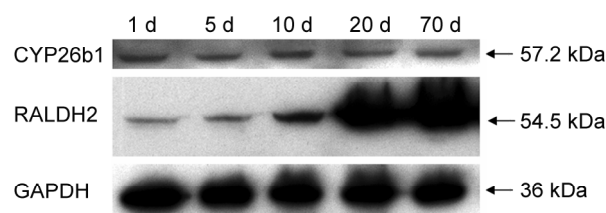


Figure 3. The quantification of RALDH2 and CYP26b1 at the protein level during mouse postnatal testis development was determined by Western-blot. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous protein for normalization. 30 μ g whole protein extracts from different ages of the mouse testes were loaded per lane.

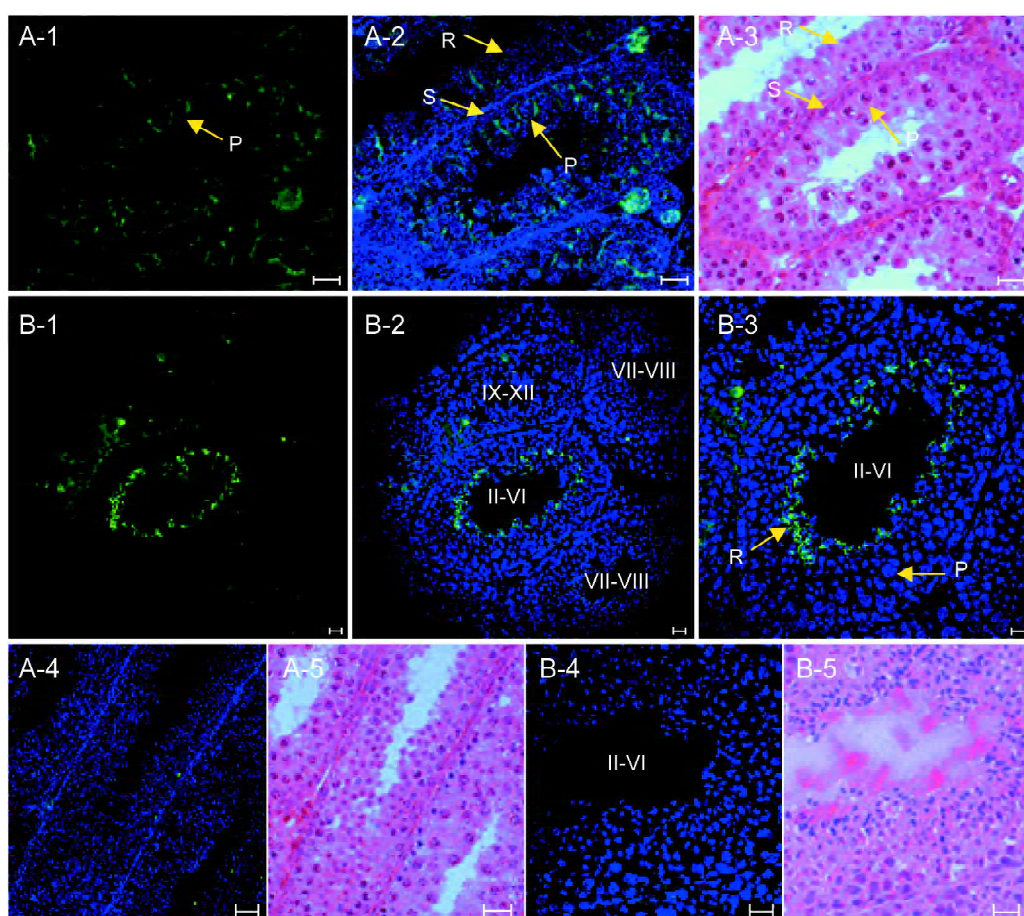


Figure 4. Expression of RALDH2 in mouse testes. The distribution of RALDH2 (green) was restricted to the cytoplasm of the pachytene spermatocytes in postnatal day 20 (A-1), while spermatogonia and round spermatids were RALDH2 negative. In postnatal day 70, round spermatids at epithelial stage II-VI expressed RALDH2 (B1-B3), while germ cells at stage VII-VIII, IX-XII and pachytene spermatocytes at stage II-VI were RALDH2 negative. Sections of the A-1 and B-1 were counterstained with DAPI (blue) as shown in A-2 and B-2. The same sections of A-1 were restained with hematoxylin and eosin as shown in A-3. B-3 was a high magnification of B-2. Negative controls of postnatal day 20 and day 70 were shown in A-4 and B-4, respectively. S, spermatogonia; P, pachytene spermatocyte; R, round spermatids. Roman numerals designate the stages of the seminiferous epithelium cycle: II-VI, stages II, III, IV, V or VI; VII-VIII, stages VII or VIII; IX-XII, stages IX, X, XI or XII. Bars = 20 μ m.

both detected in mouse testes from postnatal day 1 to adulthood. The amount of CYP26b1 protein level did not change significantly during mouse postnatal testis development, which was in agreement with their relative mRNA level, as shown in Figure 1. The RALDH2 protein level began to increase at postnatal day 10 and increased distinctly from postnatal day 20 to adulthood, which was consistent with their relative mRNA level.

3.4 Expression of RALDH2 and CYP26b1 in developing and adult mouse testis

Using indirect immunofluorescence, RALDH2 pro-

tein was undetectable in the 1, 5, and 10 day testes (data not shown). It only became detectable at postnatal day 20 in pachytene spermatocytes, whereas spermatogonia and round spermatids were RALDH2 negative. Robust fluorescent signals were observed in round spermatids at epithelial stage II-VI in adult mouse testis, whereas germ cells at epithelial stage VII-VIII, IX-XII and pachytene spermatocytes at epithelial stage II-VI were RALDH2 negative (Figure 4). The distribution of CYP26b1 protein was restricted to the cytoplasm of the peritubular myoepithelial cells of almost all seminiferous tubules both in the developing testes (5, 10 and 20 day) and adult tes-

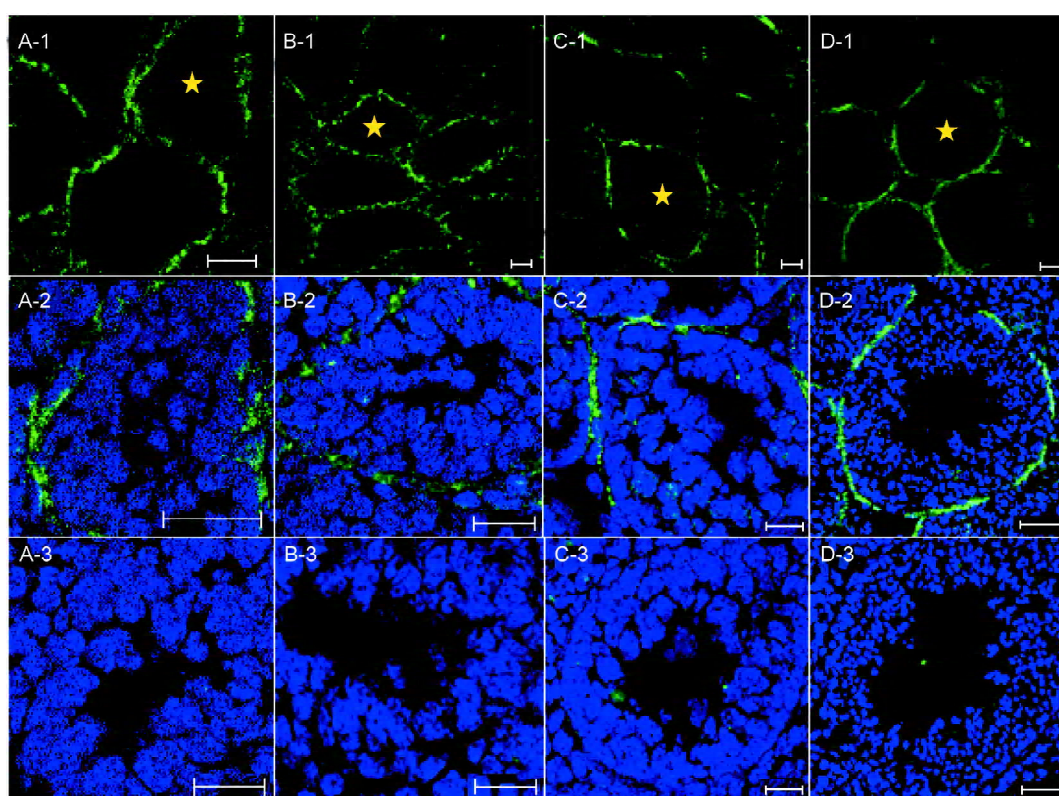


Figure 5. Expression of CYP26b1 at different ages of mouse testes. The distribution of CYP26b1 (green) was restricted to the cytoplasm of the peri-tubular myoepithelial cells at postnatal day 5 (A-1), day 10 (B-1), day 20 (C-1) and day 70 (D-1). Sections were counterstained with DAP-I (blue). High magnifications of the tubules marked with yellow star in A-1, B-1, C-1 and D-1 were showed in A-2, B-2, C-2 and D-2, respectively. Negative controls of postnatal day 5, day 10, day 20 and day 70 were showed in A-3, B-3, C-3 and D-3, respectively. Bars in A-1–A-3, B-1–B-3 and C-1–C-3 are 20 μ m; Bars in D-1–D-3 are 50 μ m.

tis (Figure 5). Negative controls using normal rabbit IgG instead of anti-RALDH2 or anti-CYP26b1 antibodies did not show any positive signals.

4 Discussion

In the work described here, the anti-RALDH2 and anti-CYP26b1 antibodies were raised successfully through immunizing rabbits with the fragments of protein RALDH2 and CYP26b1 obtained from the prokaryotic expression system. The specificity of the antibodies was satisfied as determined by Western blot. We have characterized the expression of the RA metabolizing enzymes RALDH2 and CYP26b1 during mouse postnatal testis development at both the mRNA and protein levels.

Using high-performance liquid chromatographic (HPLC) assay [15] or liquid chromatography/MS/MS

(LC/MS/MS) assay [16], some researchers have measured the RA quantity in the adult mouse whole testis extracts. However, for determining the RA level in the seminiferous tubules, using HPLC or LC/MS/MS is almost impossible, because it is very difficult to separate the seminiferous tubules from the Leydig cells existing among the seminiferous tubules, which can also produce RA through RALDH1 [10] under dim yellow light to prevent the photoisomerization and photodegradation of RA. Therefore, we tested the quantification of RALDH2 and CYP26b1 at both mRNA and protein levels, to potentially reflect the RA level in the seminiferous tubules.

During mouse testis postnatal development, the variation of *Aldh1a2* mRNA level was consistent with its protein level as shown in Figures 1 and 3, respectively. They were both increased slightly at postnatal day 10 and in-

creased obviously from postnatal day 20 to adulthood. It seemed that *Aldh1a2* transcripts decreased at postnatal day 5, while its protein RALDH2 did not exhibit such a phenomenon. This was probably due to the individual difference among the mice at postnatal day 5 used for RNA extraction. Which cell type expresses RALDH2 in testis? To test this, we used an immunohistochemistry (IHC) assay to determine RALDH2 localization in different ages of mouse testes. To our surprise, RALDH2 did not begin its expression until postnatal day 20 in pachytene spermatocytes using IHC, while it was detected from postnatal day 1 to adulthood using Western blot analysis. The discrepancy could be explained by the sensitivity difference between these two methods. It was reported that *Aldh1a2* transcripts were detected only in germ cells [10]. At postnatal days 1 and 5, the germ cells in the seminiferous tubules are only mitotic germ cells (gonocytes of 1 day and spermatogonia of 5 days), and at postnatal day 10, the most advanced germ cells are leptotene spermatocytes [17]. We are not sure whether these germ cells express RALDH2 at the early stages of testicular development based on our IHC data. At postnatal day 20, RALDH2 was detected in pachytene spermatocytes, while in adulthood it was expressed in round spermatids. This is probably because the gene expression and protein synthesis in germ cells are different at various postnatal stages. Other studies report that germ cell nuclear factor is confined in spermatogonia from postnatal day 8 to 14, but then disappears from day 17 on, and is localized in round spermatids and pachytene spermatocytes from day 28 to day 420 [18]. However, the molecular mechanism controlling the stage specific expression remains to be clarified.

In the developing and adult testis, the amount of *cyp26b1* transcripts and CYP26b1 protein did not change significantly and the CYP26b1 protein was confined to the peritubular myoepithelial cells, similar to the localization of its mRNA transcripts [10]. *Cyp26b1* transcripts seemed to decrease slightly at postnatal day 5 (similar to *Aldh1a2* transcripts), which could also be explained by the individual difference among the mice at postnatal day 5 used for RNA extraction. It was reported that CYP26b1 functioned as the meiosis inhibitor in the embryonic testis to prevent RA (the meiosis initiation factor) produced by mesonephroi reaching the Sertoli cells or the germ cells [19, 20]. Because of this, male embryonic germ cells undergo G₀/G₁ mitotic cell cycle arrest, and meiosis does not begin until postnatal day 10 in mice [17]. Our

research found that RALDH2, the RA synthesizing enzyme in the seminiferous tubules began to increase at postnatal day 10, whereas CYP26b1, the RA degradation enzyme did not change significantly during postnatal testis development, which infers that the level of RA in the seminiferous tubules might begin to increase at postnatal day 10. These results suggest that the RA signaling could be related to the initiation of meiosis in mouse testis, as reported by Baltus *et al.* [21].

Taking collectively the relative quantification of RALDH2 and CYP26b1, both at mRNA and protein levels, and the distribution of the respective proteins, our results indicate that following birth, the level of RA synthesized by RALDH2 in the seminiferous epithelium begins to increase at postnatal day 10, and maintains a high level through postnatal day 20 to adulthood.

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Contact: Phone: +86-21-5492 2824

Fax: +86-21-5492 2825

E-mail: apfa@sibs.ac.cn

aja@sibs.ac.cn

aja@mail.shcnc.ac.cn

Website: www.asiaandro.com/3APFA

