Gonadotropin Regulation of Retinoic Acid Activity in the Testis

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Abstract- Initiation of spermatogenesis in primates is triggered at puberty by an increase in gonadotropins; i.e., follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Prior to puberty, testis of the monkey contains only undifferentiated germ cells. However, spermatogonial differentiation and spermatogenesis may be initiated prior to puberty after stimulation with exogenous LH and FSH. Retinoic acid (RA) signaling is considered to be a major component that drives spermatogonial differentiation. We were interested in evaluating the relative role of LH and FSH, either alone or in combination, in regulating the retinoic acid signaling in monkey testis. Sixteen juvenile male rhesus monkeys (Macaca mulatta) were infused with intermittent recombinant single chain human LH (schLH) or recombinant human FSH (rhFSH) or a combination of both for 11 days. We then analyzed the expression of the several putative RA signaling pathway related genes; i.e., RDH10, RDH11, ALDH1A1, ALDH1A2, CYP26B1, CRABP1, CRABP2, STRA6, STRA8 in the testis after 11 days of stimulation with vehicle, LH, FSH and combination LH/FSH using quantitative real-time PCR (qPCR). The qPCR results analysis showed that administration of gonadotropins affected a significant change in expression of some RA signaling related genes in the monkey testis. The gonadotropins, either alone or in combination dramatically increased expression of CRABP2 ($P \leq 0.001$), whereas there was a decrease in ALDH1A2 expression ($P \leq 0.001$). Moreover, combined gonadotropin treatment led to the significant decrease in CRABP1 expression ($P \leq 0.05$). These findings are the first evidence that the activity of retinoic acid signaling in the monkey testis is regulated by gonadotropins (LH/FSH) levels.

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Keywords: LH and FSH; Retinoic acid; Rhesus monkey; Spermatogenesis

Introduction

The initiation of spermatogenesis in highly evolved primates is triggered at puberty by an increase in the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) after a protracted hypogonadotropic phase of juvenile development (1,2). In the rhesus monkey, this phase of prepubertal development typically extends from 6 to 36 months of age, and the seminiferous cords of the juvenile testis contain only Sertoli cells and undifferentiated spermatogonia (3). Based on nuclear staining patterns, two major types of undifferentiated spermatogonia have been identified in the rhesus monkey and other highly evolved primates (4-6). Type A dark spermatogonia (Ad) are recognized by dense and homogenous chromatin, whereas Type A pale spermatogonia (Ap) exhibit a more granular pattern of nuclear chromatin. In classical studies using histochemical markers of mitosis and thymidine labeling, Clemont proposed that Ad were "reserve" stem cells and Ap were "renewing" stem cells (7). Ad and Ap are found in approximately equal numbers in the prepubertal and adult testis of the rhesus monkey, and during juvenile development, these undifferentiated spermatogonia proliferate at a relatively slow rate in a gonadotropin-independent manner (5).

Spermatogonial differentiation in the monkey (the production of differentiating B1 spermatogonia from Ap) normally occurs at the onset of puberty in association with the increase in gonadotropin secretion at this stage of development, but this critical step in spermatogenesis may be induced prematurely by experimentally imposing a "pubertal" gonadotropin stimulus during juvenile development.

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The earlier experiment by Plant's laboratory has revealed that treatment of juvenile monkeys (16-19 months of age) for 11 days with pulsatile combined human FSH and single chain LH resulted in generation of differentiated type B spermatogonia (B1-4) and early meiotic germ cells; preleptotene (PL) and leptotene (L) spermatocytes (8). Whereas administration of pulsatile FSH and LH alone resulted in few differentiating type B1 spermatogonia (1.3 per cross section) and only undifferentiated type A spermatogonia, respectively (8). In the adult rhesus monkey, it takes approximately 1 cycle of the seminiferous epithelium (10.5 days) for an Ap to progress through the four generations of differentiating spermatogonia (B1-B2-B3-B4) and generate early meiotic spermatocytes (9,10). If the kinetics of these early steps of spermatogenesis are similar in the juvenile prematurely exposed to adult levels of gonadotropin, then it seems that Ap spermatogonia of the prepubertal monkey testis are able to immediately respond to signals resulting from the activation of somatic cells by exogenous LH and FSH stimulation (8).

Spermatogonial differentiation and later events in spermatogenesis depend on vitamin A, also known as retinol (ROL) (10). As originally demonstrated in rodents, spermatogenesis in vitamin A deficient (VAD) animals is blocked at the step of spermatogonial differentiation (11). The seminiferous tubules in such animals contain only Sertoli cells and undifferentiated type A spermatogonia (12,13). Conversely, when VAD animals were exposed to exogenous ROL, spermatogonial differentiation and spermatogenesis were restored (11,14,15,16).

Vitamin A is transported in the peripheral circulation by retinoid binding proteins (RBPs) (17), and, in the case of the Sertoli cell, the cellular uptake of vitamin A is facilitated by a membrane-bound receptor known as stimulated with retinoic acid 6 (STRA6) (18,19). The metabolism of vitamin A to all-trans retinoic acid (atRA), the active metabolite, is a two-step enzymatic process (20). Retinol is converted into retinaldehyde (RAL) by retinol dehydrogenases (RDH; eg, RDH10 and RDH11) (20,21), and then RAL is converted into RA by one of 3 retinaldehyde dehydrogenases (RALDHs), namely RALDH1 (22), RALDH2 (23), and/or RALDH3 (24); also known as ALDH1A1, ALDH1A2, and ALDH1A3, respectively. Within target cells, RA is bound to the cellular RA-binding proteins, CRABP1 and CRABP2 (25,26). CRABP2 shuttles RA to the nucleus thus facilitating the binding of RA to retinoic acid receptors (RARs) (27,28) and thereby induces RA responsive genes such as STRA8 (29). CRABP1 promotes the cytoplasmic degradation of RA via the cytochrome P450 family 26 (CYP26) enzymes, CYP26A1, CYP26B1, and CYP26C1 (15,30).

It has been suggested that gonadotropins may trigger the differentiation of spermatogonia and their meiotic entry through regulation of RA signaling in the seminiferous tubules of the testis, which provides a novel working hypothesis on the mechanisms of gonadotropins to control spermatogenesis via RA signaling (31). In the present study, we sought to determine whether exogenous LH or FSH stimulation governs intra-cellular RA activity in the testis for the first time. We have examined the differential expression pattern of several putative RA-related signaling genes using testicular RNA that had been generated from an earlier experiment in which juvenile rhesus monkeys had received intermittent intravenous infusions of single chain human LH (schLH) and recombinant human FSH (rhFSH), either alone or a combination, for 11 days (8).

Materials and Methods

Testicular RNA samples

The RNA samples for the present experiment were generated during an earlier study in which juvenile (16-19 months of age) male rhesus monkeys (Macaca mulatta) received intermittent intravenous infusions (2 ml for 1 min, every 3 h) of single chain human LH (schLH; 3 IU/kg) and recombinant human FSH (rhFSH; 2 IU/kg), either alone or in combination, for 11 days (8). The vehicle used to administer the gonadotropin treatments was DPBS. Four animals were studied in each treatment group including vehicle. At the time of castration, a fragment of testicular tissue was snap-frozen in liquid nitrogen for total RNA extraction by the single-step guanidium thiocyanate-phenol-chloroform method (32). The integrity of RNA was determined at the time of extraction by visualizing ethidium bromide-stained 28S and 18S ribosomal RNA bands with electrophoresis on agarose gel, and concentration was determined by measuring absorbance at 260 nm (33). In the present study, levels of STAR mRNA were also measured as an internal control to exclude the possibility of degradation in the RNA during the extended period of storage.

LH stimulation, either alone or in combination with FSH, resulted in an increase in testicular testosterone secretion that resulted in adult levels of the circulating steroid, and combined gonadotropin stimulation resulted in the initiation of spermatogenesis as evidenced by the

appearance of differentiating B spermatogonia and preleptotene and leptotene-zygotene spermatocytes in the testis of these animals (8). FSH treatment alone, but not that of LH, resulted in the appearance of an occasional differentiating B spermatogonia (8).

Reverse transcription PCR and cDNA synthesis

Testicular RNA (250 ng) from each of the gonadotropin or vehicle-treated animals was reverse transcribed using random hexamers (Invitrogen). The reaction mixture included 7.5 mM $MgCl_2$, 400 µM deoxynucleotide triphosphates (dNTPs; Fisher Scientific Inc., Pittsburgh, PA, USA), GeneAmp® 10X PCR Buffer II (Life Technologies, Grand Island, NY, USA), 40 U of RNasin Ribonuclease Inhibitor (Fisher Scientific Inc., Pittsburgh, PA, USA), 2.25 µM random hexamers, 250 U Superscript III Reverse Transcriptase) and nuclease free water (Life Technologies). Parallel reactions were performed without reverse transcriptase. Incubation of samples was 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)

The relative expression of several genes implicated in intra-cellular RA signaling; i.e., RDH10, RDH11, ALDH1A1, ALDH1A2, CYP26B1, CRABP1, CRABP2, STRA6, and STRA8 were analyzed with Real-time PCR. The amplifications were performed as described previously (34) in 96 well plates (MicroAmp® Fast Optical 96-Well Reaction Plates; Life Technologies) using the ABI Prism 7900HT Sequence Detection System v2.3 (Applied Biosystems, Life Technologies). Reactions, in a total volume of 20 µl, included 2 µl cDNA, 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems) and 2 µl (1 µM) of each primer. Samples with no cDNA were included in the amplification reactions as negative controls, either from reverse transcription reactions lacking either reverse transcriptase or mRNA template. Primer pairs (Table 1) were positioned with intervening introns to avoid false positives and independently validated for use in the ΔΔCt method of gene expression analysis (35) through use of a standard curve derived from serial dilutions of the cDNA obtained from the reverse transcription reactions. Primers with an efficiency of 2±0.2 were considered acceptable. The housekeeping gene VPS29 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 to characterized relative fold changes between treatment groups. For each transcript, fold-change was normalized to vehicle control (=1). The means (±SE) of three individual experiments were determined for each treatment group for each gene of interest.

Table 1. Primers used for qPCR

Transcript	Forward (5'-3')	Reverse (5'-3')	Region amplified
RDH10	CATCAACACGCAGAGCAACG	GCAAGTTACAGTGGGGCAGA	333-463
RDH11	TTTAGCTTGCCGGGATGTGG	GAGGTGCTTTTCCTCGGCTA	258-414
ALDH1A1	GCACTGAGCTGTGGAAACAC	CCTGCTGTAGGCCCATAACC	742-884
ALDH1A2	TTGCAGGGTGTCATCAAAAC	ACACTCCAATGGGTTCATGTC	337-457
CYP26B1	CACACAGGGCAAGGACTACT	GAAGATCAGCTCCAGGGTCC	568-682
CRABP1	ATCCACTGCACGCAAACTCT	TGGTACAGACCACGTCATCG	359-473
CRABP2	ACCTCGTGGACCAGAGAACT	CTACCCGTGGTCACTCACTC	329-438
STRA6	TGACAGGGACGGCCATTTAC	CACCTCCTGCTTGTCCTCTG	920-1068
STRA8	GGCCTTAGCTGTGCAAACAC	ATCTCTTCGTCAACAGGAAAGG	880-939
StAR	TGGGCATCCTTAGCAACCAG	CACTCCCCCATTGCTTCCAT	468-648
VPS29	GACTCTGGCTGGTGATGTTCA	GGGCTAAGCTGGCCATATCT	236-387

The housekeeping gene VPS29 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 to characterized relative fold changes between treatment groups. For each transcript, fold-change was normalized to vehicle control (=1). The means (±SE) of three individual experiments were determined for each treatment group for each gene of interest.

Statistical analysis

qPCR Results were analyzed after log transformation (36) by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test to

determine the statistical significance of difference at a 95% confidence interval using GraphPad PRISM version 6. 0f (GraphPad Software, San Diego, CA). The difference was considered significant at $P \leq 0.05$.

Results

Expression of StAR

As shown in Figure 1, stimulation of the juvenile monkey testis for 11 days with LH, either alone or in combination with FSH, resulted in a 6 and 5-fold increase, respectively ($P<0.05$) in the levels of the mRNA encoding StAR when compared to vehicle treatment. Although not significant, the level of the StAR mRNA in testes stimulated by FSH, alone, was 2-fold higher than that in control (Figure 1).

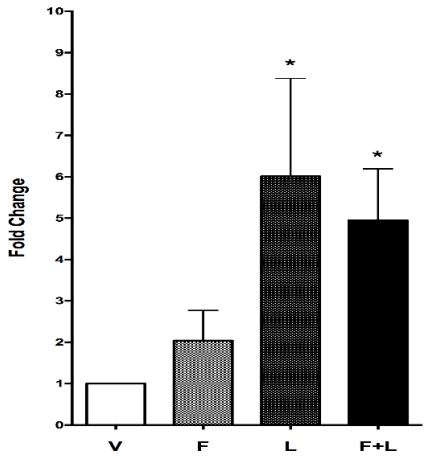

Figure 1. Expression levels of the StAR gene in the monkey testis. Values are the means (±SE) of four independent experiments of 11 days of pulsatile infusion of vehicle (V), rhFSH (F), schLH (L), or a combination of the two gonadotropins (F+L). Significant differences ($P \leq 0.05$) from vehicle are denoted with an asterisk (*)

Expression of genes encoding RA-synthesizing and degradation enzymes

Of the five studied encoding proteins that are involved in the regulation of RA synthesis and degradation (RDH10, RDH11, ALDH1A1, ALDH1A2, and CYP26B1) only ALDH1A2 was affected by gonadotropin treatment (Figure 2). Stimulation with LH and FSH, either alone or in combination, resulted in an approximately 50% decrease in *ALDH1A2* mRNA levels ($P<0.05$). In contrast, the differences between LH or FSH alone and LH+FSH were not significant (Figure 2).

Expression of genes encoding RA-binding and target proteins

CRABP2 mRNA levels were increased by approximately 3 fold ($P<0.05$) with FSH and LH stimulation, alone, and combined gonadotropin treatment resulted in 4- to 5-fold increase, but this synergistic effect was not significant (Figure 3). *CRABP1* mRNA levels were decreased significantly ($P \leq 0.05$) only after combined FSH and LH stimulation (Figure 3).

Examination of the transcripts encoding the RA downstream target proteins, STRA6 and STRA8, indicated that expression of STRA6 was increased by 2- to 3-fold by all 3 gonadotropin treatments, but the effect was not significant (Figure 3). STRA8 mRNA levels were not altered by the hormone treatments (Figure 3).

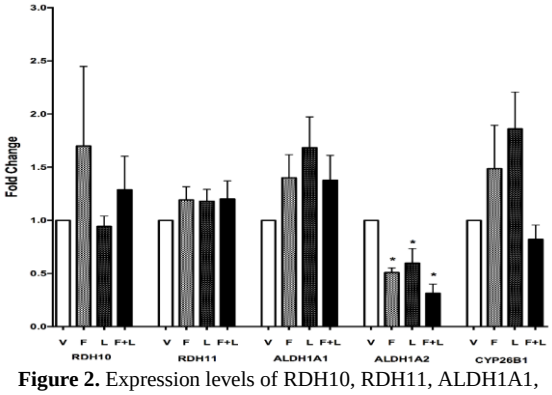

Figure 2. Expression levels of RDH10, RDH11, ALDH1A1, ALDH1A2, and CYP26B1 gene in the monkey testis. Values are the means (±SE) of four independent experiments of 11 days of pulsatile infusion of vehicle (V), rhFSH (F), schLH (L), or a combination of the two gonadotropins (F+L). Significant differences ($P \leq 0.001$) in the expression of ALDH1A2 from the vehicle are denoted with an asterisk (*).

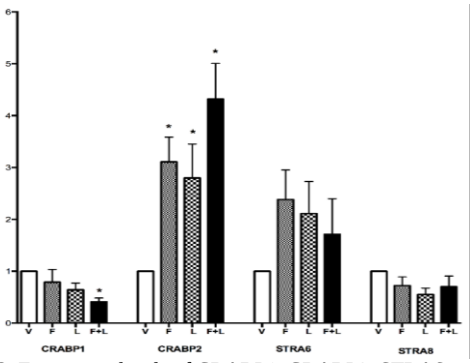

Figure 3. Expression levels of CRABP1, CRABP2, STRA6, and STRA8 gene in the monkey testis. Values are the means (±SE) of four independent experiments of 11 days of pulsatile infusion of vehicle (V), rhFSH (F), schLH (L), or a combination of the two gonadotropins (F+L). Significant differences in expression of CRABP1 and CRABP2 genes from the vehicle are denoted with an asterisk (*) ($P \leq 0.05$ and $P \leq 0.001$ respectively).

Discussion

As stimulation with gonadotropins, LH either alone or in combination with FSH, enhances the expression of steroidogenic acute regulatory (StAR) and steroidogenesis in the testis (37,38,39), the concomitant and dramatic up-regulation in testicular *StAR* mRNA expression was to be expected. Therefore, up-regulation in testicular *StAR* gene by gonadotropins provided evidence to indicate that the integrity of the testicular RNA had not been compromised.

Our results showed that there are no significant differential effects of LH and FSH either alone or in combination on the expression of the studied RA pathway related genes RDH10, RDH11, ALDH1A1, CYP26B1, STRA6 and STRA8 after 11 days. Whereas, the expression of ALDH1A2 and CRABP1 were significantly down-regulated and CRABP2 was up-regulated by gonadotropins stimulation.

A previous comprehensive study by Vernet *et al.*, (2006) investigated the expression and localization of RA-related signaling genes in the developing and adult mouse testes and revealed that Aldh1a1 and Aldh1a3 proteins are expressed in Leydig and Sertoli cells (40). Whereas, *Aldh1a2* transcript was expressed in a stages specific manner (VII-XI) in pachytene spermatocytes after postnatal day 20 (40).

Testis has intrinsic ability to produce RA (41,42). The spermatogonial stem cells (SSCs) niche outside of the seminiferous tubules is thought to regulate spermatogonial divisions and proliferation. It has been found that ALDH1A2 expression in the interstitium of the juvenile testis, specifically by peritubular macrophages which are associated with positions of the seminiferous tubule containing undifferentiated spermatogonia, is critical for juvenile spermatogenesis (43). It has been suggested that ALDH1A2, but not ALDH1A1 and ALDH1A3, is the main enzyme involved in RA biosynthesis in human germ cells, and relevant protein levels correlate with the number of germ cells and male infertility (44). Localization of ALDH1A2 protein was found in undifferentiated spermatogonia, spermatocytes, and spermatids in the human testis (45). The expression of ALDH1A2 was down-regulated across the development of human fetal testis (42). Down-regulation of ALDH1A2 after 11 days of gonadotropin stimulation led us to suggest that ALDH1A2 might be responsible for self-renewal and proliferation of spermatogonia in the juvenile testis. However, the precise role of ALDH1A2 in the monkey testis and downregulation during this important developmental time point of spermatogonial differentiation needs to be investigated further.

It has been confirmed that the expression of the RA metabolizing enzyme Cyp26b1 in the premature testis shields germ cells from the meiosis-inducing action of RA. There is no extra-tubular source of RA in the adult mouse testis because exogenous sources of RA are insulated from the seminiferous epithelium through the expression of CYP26 in the peritubular myoid cells (40). These observations led Vernet *et al*., (2006) to suggest that cells within the seminiferous epithelium are responsible for synthesizing and controlling of RA levels.

CRABP I and II are exclusively intracellular proteins (46), and it has been demonstrated that these RA-binding proteins play important roles to control the actual level of intracellular RA (47). Previous studies have revealed that the expression pattern of CRABP1 and CRABP2 has been conserved between rat and mouse testis (40,48,49). In the mouse testis, *CRABP1*, which is thought to target RA for degradation, was expressed only in the spermatogonia, but not in other germ cell and somatic cells (40). It has been revealed that CRABP-I is exclusively localized to the cytoplasm of embryonic gonocytes and spermatogonia of the post-natal and adult testis but not in Sertoli cells (48,49). The exclusive expression of CRABP-I in the cytoplasm of gonocytes and spermatogonia indicate possible role of CRABP-I in degradation RA in these actively dividing germ cells, outside the blood-testis barrier mediated by Sertoli cells, and thereby preventing activation of retinoic acid receptor in the nucleus (46,49).

In contrast to Crabp-I, the expression of Crabp-II, which promotes RA signaling, was detected in only Sertoli and Leydig cells in the rat fetus testis (46,49). *Crabp-II* mRNA was detected as high levels at postnatal days in the rat testis (49). This expression pattern of CRABP-II in fetal and prepubertal Sertoli cells which correlates with the developmental timing of Sertoli cell proliferation led Zheng *et al*., (1996) to propose that CRABP-II is involved in the RA-dependent autocrine or paracrine regulation of Sertoli cell proliferation (49). The expression of Crabp-II in Sertoli cells led Zheng *et al*., (1996) to suggest that Sertoli cell might be the site of RA synthesis within the seminiferous tubules of the testis and it is essential in the RA-dependent up-regulation of Sertoli cell proliferation (49).

It has been demonstrated that CRABP-II, but not CRABP-I, is essential to direct channeling of RA to the RARα receptor in the nucleus (50,51). While CRABP-I has been possibly implicated in inactivation of RA,

CRABP2 promotes the nuclear transfer of RA and hence is important for the biological effects of RA. It also has been suggested that CRABP-II acts as a molecular mediator of RA activity (52). Previous studies have revealed that overexpression of CRABP-II was involved in human germ cell tumor differentiation (53). A recently study demonstrated that expression of CRABP-I inhibits RA signaling by degrading RA in the cytoplasm, and proposed RA-binding proteins CRABP-I and CRABP-II as biomarkers for predicting cell response to RA, so that elevated levels of cytoplasmic CRABP-I associated with RA resistance, and elevated levels of nuclear CRABP-II associated with sensitivity to RA (54).

Based on previous published observations and down-regulation of CRABP1 after 11 days of combined hormone stimulation, we suggest that CRABP1 might be responsible for degradation of RA within undifferentiated spermatogonia in juvenile monkey testis and thereby prevents spermatogonia differentiation and initiation of spermatogenesis in the seminiferous tubules of the juvenile monkey testis. Whereas, up-regulation of CRABP2 after either hormone treatment alone or combined hormone stimulation led us to suggest that CRABP2 might be responsible for activation of RA signaling in the Sertoli cells within seminiferous tubules of the monkey testis and thereby provides paracrine factors which are necessary for initiation of spermatogonia differentiation and their meiotic entry.

Our results show that after 11 days of gonadotropins stimulation there was no significant change in the expression of RA-responsive genes STRA6 and STRA8. It has been reported that Stra6 is localized only in the plasma membrane of the basal pole of Sertoli cells in the adult mouse testis in the same tubules which expressing Stra8 (18), while STRA8 which is required for their meiotic entry (29,55), is only expressed in the differentiating spermatogonial population (55). It has been demonstrated that activation of RA signaling occurs only within discrete patches of neonatal murine testicular tubules, which suggest the performance of RA signaling in these particular patches at the initiation of spermatogenesis (56).

It has been demonstrated that the expression of *STRA8* is absent in human gonad testis at all examined gestations; however, RA has induced the *STRA8* expression in the human fetal testis (42). In a previous study, the expression of STRA8 was not detected in the human fetal testis (57); whereas**,** STRA8 was weakly detected in the nuclei of small subset of differentiating B spermatogonia and spermatocytes in the human adult testes (57). These observations led us to suggest that the expression pattern of STRA8 in primate testis may be different from that reported in mice. However, the expression of STRA8 remains unexplained and needs further studies.

In summary, the differential expression pattern of RA signaling related genes in the monkey testis after stimulation with gonadotropins provides the novel finding that implicates gonadotropins FSH and LH as key factors involved in the regulation of RA activity in the initiation of spermatogonia differentiation and spermatogenesis in the monkey testis. The overall monkey expression pattern of RA signaling related genes indicates an important role of gonadotropins in regulating the spermatogonia renewal–differentiation switch presumably through regulation of CRABP1 and CRABP2 expression. Taken together, gonadotropins regulation of RA signaling during spermatogenesis, which has not been previously reported, needs further studies to establish the exact mechanism.

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