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INVITED REVIEW

Sperm Biology

The role of retinoic acid in the commitment to meiosis

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Male meiosis is a complex process whereby spermatocytes undergo cell division to form haploid cells. This review focuses on the role of retinoic acid (RA) in meiosis, as well as several processes regulated by RA before cell entry into meiosis that are critical for proper meiotic entry and completion. Here, we discuss RA metabolism in the testis as well as the roles of stimulated by retinoic acid gene 8 (STRA8) and MEIOSIN, which are responsive to RA and are critical for meiosis. We assert that transcriptional regulation in the spermatogonia is critical for successful meiosis.

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INTRODUCTION

Entry into male meiosis is a complex process regulated by several important signals. In this review, we highlight the importance of retinoic acid (RA) in spermatogenesis with specific emphasis on the commitment of germ cells to meiosis in the testis. We expand on several signaling pathways influenced by RA and how successful meiotic entry and completion is influenced far before the formation of preleptotene spermatocytes. Our primary focus is on data generated from rodent studies, though with implications for human fertility.

THE ROLE OF RA IN SPERMATOGENESIS

All-*trans* retinoic acid (hereafter referred to as RA) is an active metabolite of vitamin A (retinol) and essential for the commitment of germ cells to spermatogenesis. Spermatogenesis is a process whereby relatively undifferentiated germ cells mature into spermatozoa within the seminiferous tubules. RA is involved with several critical processes to achieve proper spermatogenesis, including spermatogonial differentiation (also termed the spermatogonial A-to-A1 transition), meiotic entry, spermiation, and blood–testis barrier formation.[1–6] The transition of undifferentiated A spermatogonia (A_{undiff}) to A1 spermatogonia marks an irreversible commitment to meiosis and the spermatogenic processes to ultimately form spermatozoa. A pulse of RA occurs every 8.6 days in the adult mouse which triggers the A-to-A1 transition.[7] This pulse of RA appears to move lengthwise along the tubules establishing a cycle in the seminiferous epithelium, with twelve stages defined in the mouse based on germ cell associations within a cross-section of a tubule and the steps of spermatid maturation (**Figure 1**).[8,9] This process has been compared to multiple waves moving through the tubules as the germ cells develop, so at any time, there are cells at various stages of development throughout the testis. This is critical for continual fertility, allowing mature spermatozoa to be constantly released into the tubule lumen.

The importance of RA for fertility was originally shown via the study of vitamin A-deficient rodents. These animals were sterile and did not contain any germ cells beyond undifferentiated spermatogonia. However, if these animals were administered an exogenous dose of either retinol or RA, spermatogonial differentiation was triggered and resulted in complete spermatogenesis.[10–13] Further research has shown that in the absence of RA, spermatogonia fail to complete the A-to-A1 transition.[13,14] For retinol-deficient mice, spermatogonial populations remain paused at the A_{undiff} stage until they receive an exogenous dose of RA which subsequently pushes the majority of A_{undiff} spermatogonia to transition to differentiating A spermatogonia.

Levels of endogenous RA have been measured throughout spermatogenesis which revealed a spike of RA concentration between Stages VII and VIII of the seminiferous epithelium, with maximum levels reached in Stages VIII–IX.[15] This coincides with the timing of the A-to-A1 transition, entry of preleptotene spermatocytes into meiosis, and spermiation. The timing of the RA pulse, as well as the previously mentioned retinol/RA deficiency studies and chemical RA manipulations shown by Endo *et al*.,[16] shows that the maintenance of RA levels throughout the cycle of the seminiferous epithelium is critical for proper spermatogenic regulation. Synthesis of all-*trans* RA in the testis, depicted in **Figure 2**, occurs through two oxidation reactions. First, retinol is shuttled to the testis with the help of retinoic acid binding protein 4 (RBP4) and transthyretin complex (TTR). Upon arriving in the testis, the retinol complex is admitted into the Sertoli cell through membrane receptors, including stimulated by retinoic acid gene 6 (STRA6).[7,17] While other receptors have been hypothesized for retinol uptake in other tissues, such as retinol binding protein receptor 2 (RBPR2) in zebrafish liver and intestine to admit retinol complexed with RBP4,[18] no testis-specific retinol receptors are as widely accepted as STRA6. Once in Sertoli cells, retinol undergoes the first oxidation step primarily using the enzyme retinol dehydrogenase 10 (RDH10) to convert retinol into retinal. The second and rate-limiting step is the oxidation of retinal to RA, which

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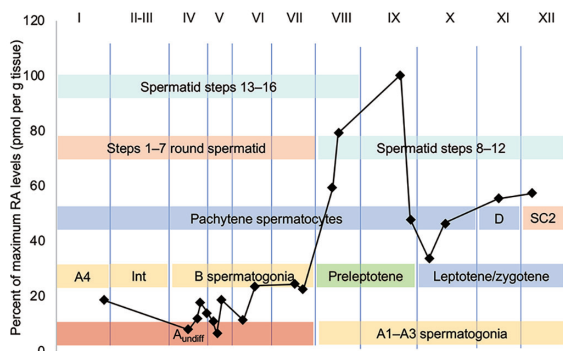

Figure 1: Level of RA throughout spermatogenesis. Depicted here are the observed levels of RA collected by Hogarth *et al.*[15] overlayed with the twelve stages of spermatogenesis with the cell types present in each stage, including undifferentiated spermatogonia (A_{undiff}), differentiating A1–A4, Intermediate (Int) and B spermatogonia, preleptotene, leptotene, zygotene, and pachytene spermatocytes, diplotene (D) and secondary spermatocytes (SC2), round spermatids (steps 1–7), and elongating spermatids (steps 8–16). RA: retinoic acid.

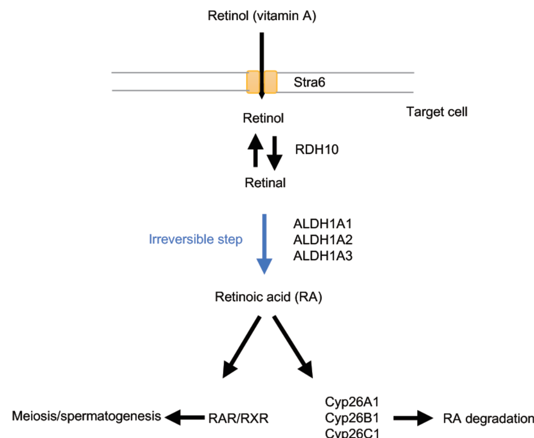

Figure 2: RA synthesis pathway. Retinol enters the target cell through the Stra6 receptor where it is oxidized via RDH10 into retinal. The second oxidation reaction is catalyzed by ALDH1A family proteins. These proteins can be inhibited by WIN 18,446 which prevents retinal's transition to RA. RA is then either used to mediate meiosis via binding to RAR/RXR transcriptional regulators or is inactivated via CYP26 family proteins resulting in meiotic arrest until a sufficient level of active RA is present. RA: retinoic acid; Stra6: stimulated by retinoic acid gene 6; RDH10: retinol dehydrogenase 10; ALDH1A: aldehyde dehydrogenase 1A; RAR: retinoic acid receptor; RXR: retinoid X receptor; CYP26: cytochrome P450, family 26 proteins.

is catalyzed by the aldehyde dehydrogenase 1A (ALDH1A) family of enzymes (further described below).

CYP26-MEDIATED DEGRADATION OF RA

Degradation of RA is especially important in developing testis tissue. Meiosis is initiated prepubertally in the testis by RA-dependent regulatory factors, such as stimulated by RA gene 8 (STRA8); however, inactivation of RA by cytochrome P450, family 26 proteins (CYP26) has been shown to inhibit *Stra8* activation and thus prevent meiotic completion.[7,19] CYP26 proteins help regulate meiosis by contributing to the degradation of RA into inactive forms, thus encouraging a delay in meiotic initiation.[7,20] There are three isoforms of the CYP26 protein found in the postnatal testis (CYP26A1, CYP26B1, and CYP26C1); however, the data largely focus on CYP26B1.[7]

CYP26B1 was observed in immature Sertoli cells of the fetal gonads serving to prevent meiotic entry.[19,21] Cell-specific studies which ablated *Cyp26a1* and *Cyp26b1* in germ and Sertoli cells found these proteins to be necessary for normal spermatogenic function. Animals without *Cyp26a1* and *Cyp26b1* in either germ or Sertoli cells displayed spermatogenic defects but retained fertility. However, ablation of *Cyp26b1* alone in both cell types simultaneously resulted in loss of mature germ cells which greatly decreased fertility, highlighting the importance of CYP26 enzymes in RA regulation and consequently fertility.[15] Since this discovery, CYP26B1 has been further characterized *in vivo*, and it has been demonstrated that A_{undiff} spermatogonia signal Sertoli cells to inhibit CYP26B1 production using NOTCH signaling. This decrease in CYP26B1 then allows RA accumulation and activation of RA-inducible genes, such as *Stra8*, and ultimately the A-to-A1 transition.[22,23] All these data support the notion that CYP26B1 suppresses entry into meiosis by reducing RA levels, consequently preventing premature activation of necessary RA-activated genes needed for meiotic initiation.

ALDH PROTEINS IN THE RA RESPONSE

As described previously, testicular all-*trans* retinoic acid is metabolized in a two-step process. Vitamin A (retinol) is converted to retinal via retinol dehydrogenase 10 (RDH10).[24] The second and rate-limiting step is the conversion of retinal to RA, which is catalyzed by the ALDH1A family of enzymes. The localization of ALDH1A1, ALDH1A2, and ALDH1A3 in the testis revealed these enzymes as likely candidates to contribute to RA synthesis.[25] While all of these enzymes have the capacity to catalyze this reaction, greater levels of ALDH1A1 and ALDH1A2 suggest that these are the best candidates to produce biologically relevant RA levels within the testis.[25] While there is a greater abundance of ALDH1A1 in the testis, the biological activity of ALDH1A2 accounts for the majority of RA synthesis during spermatogenesis.[25] ALDH1A1 is found in Sertoli and peritubular myoid cells, while ALDH1A2 is present in peritubular myoid cells and most notably in germ cells, specifically pachytene spermatocytes and round spermatids.[26] The protein expression of ALDH1A2 appears to shift slightly during prepubertal development before stabilizing in adulthood.[26] While ALDH1A3 is predicted to be a minor contributor to RA synthesis in the testis, protein expression was detected in both germ and Sertoli cells.[25] Interestingly, while RA levels respond in a pulsatile manner during the cycle of the seminiferous epithelium, ALDH1A levels and activity do not fluctuate over the course of the cycle.[26] This indicates that while ALDH1A enzymes are important to synthesize RA, these enzymes do not appear to regulate the timing of the RA pulse.

Chemical inhibition and genetic knockout (KO) studies of the ALDH1A enzymes have provided valuable insight into enzyme responsibility for RA synthesis *in vivo*. WIN 18,446 is an effective inhibitor of the ALDH1A enzymes.[27–30] When this chemical is administered daily before the endogenous RA pulse, spermatogonial differentiation is inhibited, resulting in a large pool of undifferentiated spermatogonia. However, once exogenous RA is administered, spermatogonial differentiation is triggered in the vast majority of undifferentiated spermatogonia. Spermatogenesis then proceeds normally, although in a synchronous manner (**Figure 3**).[30] This method has allowed for the isolation of purified cell populations to work around the natural cellular heterogeneity in the testis, including fluorescence-activated cell sorting of germ cells as recently described.[31] However, if an endogenous RA pulse occurs before treatment with WIN 18,446, inhibition of the ALDH1A enzymes does not occur immediately.[32] Instead, spermatogenesis proceeds for several more rounds before degenerative tubules are observed.

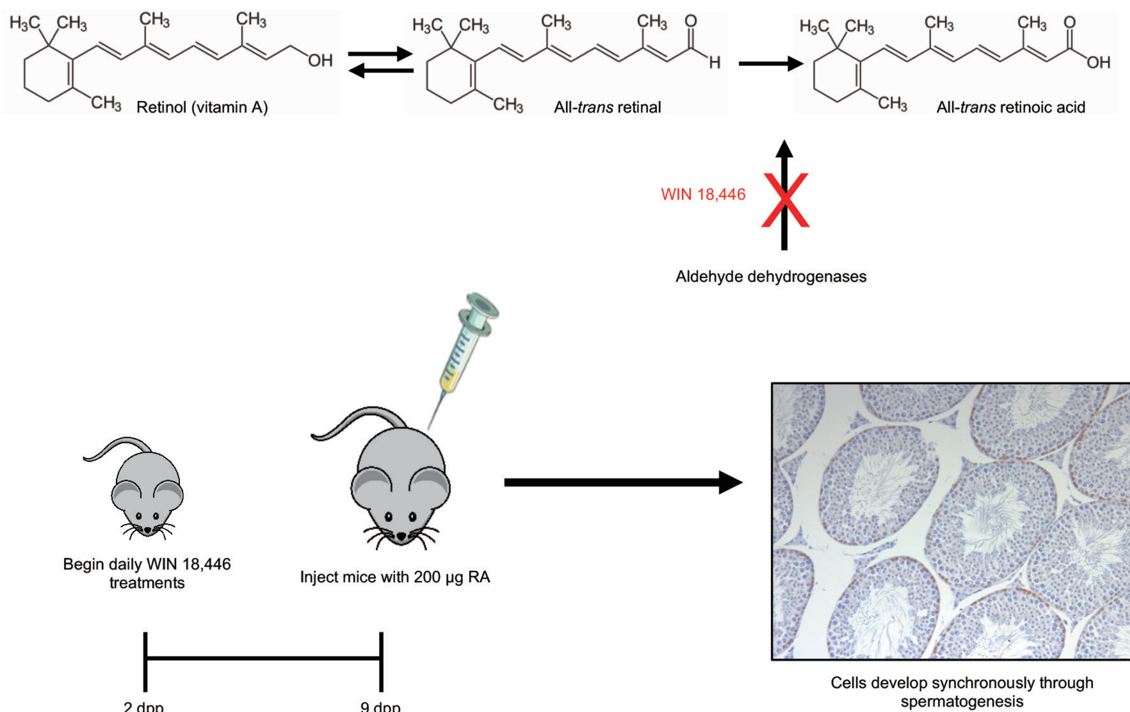

Figure 3: WIN 18,446 treatment schematic. WIN 18,446 blocks the conversion of retinal to retinoic acid by inhibiting the ALDH1A enzymes. Mice are fed 100 mg kg^{-1} of WIN 18,446 from 2–8 days postpartum (dpp). At 9 dpp, an RA injection is given to stimulate synchronous spermatogenesis, resulting in only a few types of cells being present along the length of the seminiferous tubules, contrasting the normal cellular heterogeneity in the testis. RA: retinoic acid; ALDH1A: aldehyde dehydrogenase 1A.

In addition to these broad class chemical studies, genetic ablation of ALDH1A enzymes has provided further insight into how these enzymes regulate testicular RA synthesis. *Aldh1a1*-null mice are both viable and fertile,[33] while *Aldh1a3*-null mice are born alive but die shortly thereafter of respiratory distress.[34] *Aldh1a2*-null mice die prenatally around embryonic day 9.5–10.5.[35] Because of the early lethality in all the genetic knockouts aside from *Aldh1a1*, studies turned to a more targeted way to delete ALDH1A enzyme function specifically within the testis. One study has shown that either a postnatal germ cell targeted deletion or a global postnatal deletion of *Aldh1a2* did not significantly reduce testicular RA levels nor cause an impact on fertility.[32] Although this study did not find an increase in mRNA levels of the other *Aldh1a* enzymes, the data suggest that other ALDH1A proteins are sufficient when another is deleted. Additionally, Raverdeau *et al.*[36] generated mice with floxed alleles of all three ALDH1A enzymes and utilized *Amh*-Cre to excise these genes from Sertoli cells. Spermatogonia in these mice were unable to efficiently undergo the spermatogonial A-to-A1 transition, and no advanced germ cell types were present. A single exogenous RA administration allowed these cells to not only transition from $A_{aligned}$ (A_{al}) to A1 spermatogonia, but they also completed meiosis and formed spermatozoa. These data showed that RA synthesized by Sertoli cells is necessary for the initial round of spermatogonial differentiation. However, RA synthesized in germ cells plays a complementary role within the testis. When *Aldh1a1–3* were selectively ablated in germ cells, spermatogonial differentiation, meiosis, and spermiation all took place similarly to those processes in wild-type (WT) mice.[37] While the germ cell contribution may seem dispensable, further study showed that germ cells did not progress beyond A_{al} spermatogonia when ablation of all three *Aldh1a* genes was achieved in both germ and Sertoli cells. However, when RA was exogenously provided to these animals, spermatogonia did not progress through spermatogenesis, in contrast to the Sertoli-only ablation where spermatogenesis was restored and mature spermatozoa were produced.[37] These data show that while the initial RA pulse comes from Sertoli cells, continuation of spermatogenesis can utilize RA from another source, likely more advanced germ cells that are present at the onset of the second round of spermatogonial differentiation.

RETINOIC ACID RECEPTORS IN THE TESTIS

After RA binds to receptors in the target cells, RA can then begin to regulate the cell through transcriptional changes.[30,38,39] In the late 1980s, RA was discovered to act as a ligand for many nuclear receptors which in turn altered gene expression.[40] Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) form heterodimers at retinoic acid response elements (RAREs) which are able to bind RA and alter expression of target genes.

There are three isoforms of RARs in mammals: RAR alpha (RARα), RAR beta (RARβ), and RAR gamma (RARγ). In mice, RARα is critical for normal testis development and is primarily localized in Sertoli cells and some early germ cells.[7] When knocked out, RARα-null mice are sterile and resemble a vitamin A-deficient phenotype.[41] Rosselot *et al.*[42] showed that expression of a dominant-negative RAR (RARDN) results in a similar phenotype as RARα KO animals. Our laboratory utilized a Sertoli-specific RARDN mouse model to investigate RAR signaling and discovered loss of mature germ cells resulting from the absence of RAR signaling (unpublished data). The RARβ isoform in rats has been identified in germ cells and linked to Sertoli cell activity, yet only low levels of RARβ have been detected in Sertoli cells.[43,44]

RARγ has largely been identified in A_{undiff} spermatogonia. RARγ KO animals remain fertile with minor changes in differentiation.[7,19,45] These RARs act as transcriptional activators when bound to RXR and RA.[39] Just as there are alpha, beta, and gamma isoforms of RARs, RXRs also have three isoforms (RXRα, RXRβ, and RXRγ). RXRα-null mice are embryonic lethal, thus making the isoform difficult to characterize.[46] When RXRβ is globally knocked out, these animals experience a delay in spermatid release and ultimately testis degradation, demonstrating the significance of RXRβ for normal spermatogenesis.

The RAR/RXR complex binds to RARE sites in the genome which in turn recruits either co-repressors or co-activators depending on their activation state. RAR/RXR interaction time with DNA has been highly debated; most studies support the notion that the heterodimer binds to DNA for longer periods of time, if not constantly when RA is absent.[39,47] Without RA, the RAR/RXR complex recruits co-repressors to downregulate transcription of target genes. However, in the presence of RA, the RAR binding domain is altered, evicting the co-repressors and recruiting co-activators in its place. Some activators act as transcription factors working to directly upregulate transcription of the target gene, while others modify the chromatin landscape for optimal transcription.[39] The locations of all these RARE sites remain largely unknown; however, a few genes have been identified as being RA-inducible, such as *Stra8* and *Rec8*, both of which are required for meiosis.[39,48,49] REC8 is a meiotic recombination protein that assists in maintaining cohesion in chromosome division. In *Rec8*-null mice, germ cells could not develop past spermatocytes, thus leaving these mice infertile.[50] Overall, these studies demonstrate the impact of RAR/RXR in regulating RA-dependent genes to support meiosis and overall fertility in the mouse model.

THE ROLE OF STRA8 IN MEIOTIC PREPARATION

One protein known to be important for meiosis is STRA8. Several studies using STRA8 KO mice have shown the necessity of this protein in meiotic initiation and completion. On a mixed background, STRA8 KO mice are unable to complete meiosis and most arrest at or before the leptotene stage.[51] Surviving germ cells undergo premature chromosome condensation and thus are unable to yield proper haploid cells from the meiotic process. Even more striking was a study using STRA8 KO mice on a congenic background, in which the majority of cells were unable to properly initiate meiosis from the preleptotene spermatocyte stage.[52] Both these studies conclude that regardless of the genetic background, mice lacking STRA8 are unable to properly complete meiosis, and thus, spermatogenesis is halted and results in infertility. While the necessity of STRA8 during meiosis has been well defined, the role of this protein before meiotic entry remains to be fully explored.

There has been conflicting evidence regarding the role of STRA8 during spermatogonial development. An increase in LIN28- and zinc finger and BTB domain containing 16 (ZBTB16)-positive spermatogonia in STRA8 KO mice, representing the A_{undiff} population, supported the notion that STRA8 aids in, but is not strictly required for, spermatogonial differentiation and development.[21,52–54] However, due to the defect in meiotic initiation and completion in STRA8 KO mice, we recently addressed how this meiotic defect may be originating during spermatogonial development.[31] We used the WIN 18,446/RA synchrony protocol to isolate spermatogonia corresponding to each stage of development from A_{undiff} to A1 through B spermatogonia and assessed the transcriptomes of both WT and STRA8 KO mice. We found that already at 12 h after RA injection when RA-induced genes were activated, there were many transcriptome differences between the WT and STRA8 KO cells. Additional differences were seen throughout the course of spermatogonial development, primarily the retention of transcripts associated with A_{undiff} cells and fewer transcripts associated with differentiating spermatogonia in the STRA8 KO cells. The STRA8 KO cells showed a lack of response to the initial RA pulse as compared to WT cells and continued to show abnormal transcriptomes during development. Interestingly, principal component analysis revealed that the transcriptomes of cells corresponding to B spermatogonia in STRA8 KO mice were more similar to A_{undiff} cells and clustered far away from WT B spermatogonia. Thus, while STRA8 may not be required for cell survival during spermatogonial differentiation and development, the lack of this protein results in abnormal spermatogonial transcriptomes before formation of preleptotene spermatocytes and entry into meiosis, perhaps acting as a precursor to the meiotic defects seen in these mice.

MEIOSIN - A NOVEL MEIOTIC REGULATOR

One protein which has more recently been shown to have meiotic importance is MEIOSIN. This protein, produced by the gene previously known as *Gm4969*, is present during both male and female meiosis.[55,56] Interestingly, MEIOSIN shows a similar expression pattern to STRA8. However, in the male, MEIOSIN is only present in spermatocytes at Stages VIII–IX where RA is present and is not expressed in spermatogonia within those same stages of the seminiferous epithelium. One long-standing question has been why spermatogonia do not enter meiosis when exposed to the RA pulse similarly to how they respond when exposed to RA as preleptotene spermatocytes. The nuclear colocalization of MEIOSIN with STRA8 in preleptotene spermatocytes may help to answer this question, as it appears both are required to initiate meiosis rather than undergoing spermatogonial differentiation when only STRA8 is present in spermatogonia. STRA8 and MEIOSIN appear to be at least somewhat independently regulated, as MEIOSIN protein is still present in STRA8 KO mice, and STRA8 protein is present in MEIOSIN KO mice. While MEIOSIN localization is nuclear in STRA8 KO animals, there appeared to be a greater percentage of STRA8 localized in the cytoplasm in MEIOSIN KO animals.[55] These data suggest that MEIOSIN may help retain STRA8 in the nucleus in WT animals to perform its noted actions as a transcription factor during meiotic initiation.[57] Additionally, MEIOSIN and STRA8 appear to both be regulated directly via RA.[55] WIN 18,446 has been previously described to robustly block RA synthesis and thus spermatogonial differentiation in the testis.[27,30] Ishiguro *et al.*[55] showed that MEIOSIN expression is also blocked when mice are treated with WIN 18,446 following spermatogonial differentiation and is initiated when mice are given an exogenous dose of RA. Overall, it appears that MEIOSIN acts in concert with STRA8 in a complex to regulate many genes involved with meiotic processes to control the timing and initiation of meiosis.

CONCLUSIONS

RA is a key factor for many spermatogenic processes, including the spermatogonial A-to-A1 transition before meiosis as well as preleptotene spermatocyte entry into meiosis. Here, we have highlighted several key regulatory processes in relation to RA, including its metabolism and how perturbations to these processes via WIN 18,446/RA can result in synchronous spermatogenesis. Further, we have highlighted how STRA8 and MEIOISIN are notable downstream RA targets which help promote spermatogonial development and completion of meiosis. Overall, we posit that RA is crucial not only for meiosis itself but also for proper testis regulation and maturation of spermatogonia which are needed for meiotic completion and ultimately fertility.

AUTHOR CONTRIBUTIONS

MCS and RLG conducted relevant literature search, writing the manuscript, and preparing the figures. MDG critically revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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