

THEMATIC REVIEW

Cellular and molecular basis for the action of retinoic acid in spermatogenesis

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Abstract

Spermatogenesis is a highly organized and regulated process that requires the constant production of millions of gametes over the reproductive lifetime of the mammalian male. This is possible because of an active stem cell pool and an ordered entry into the germ cell developmental sequence. The ordered entry is a result of the synthesis and action of retinoic acid allowing for the onset of spermatogonial differentiation and an irreversible commitment to spermatogenesis. The periodic appearance and actions of retinoic acid along the seminiferous tubules is a result of the interactions between germ cells and Sertoli cells that result in the generation and maintenance of the cycle of the seminiferous epithelium and is the subject of this review.

Key Words

- ▶ retinoic
- ▶ spermatogenesis
- ▶ Sertoli cells
- ▶ spermatogonia

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Introduction

Spermatogenesis is the development of the male gamete in the testis. It is a unique process in that there is an extended requirement for an active stem cell population that develops into morphologically distinctive motile spermatozoa over a time period that may be 40–70 days. In addition, the number of sperm required to achieve fertility is very large. In humans, it is estimated that about 1500 sperm cells are made every second and from 15 to 200 million sperm per milliliter of semen are required for fertility. In many mammals, fertility may extend from puberty throughout the lifetime of the male. Thus, spermatogenesis has evolved mechanisms for the expansion of cell numbers and for staggered development that assures a constant supply of the required number of gametes (Dym *et al.* 1979, 1987, Sharpe 1994, Eddy 1998, Sharma 2011). A primary system that has evolved to control these mechanisms is the synthesis and degradation of, and signaling by, retinoic acid (RA) (Griswold 2016).

Spermatogenesis can be described by three major biological steps that include: (1) the renewal of stem cells and the creation of and amplification of numbers of progenitor cells by mitosis; (2) the reduction, by one-half, of the number of chromosomes in each progenitor cell by meiosis; and (3) the differentiation of haploid cells into spermatozoa by spermiogenesis (Sharma 2011). In the mouse, the first progenitor cells derived mitotically from the stem cell population are considered ‘undifferentiated’ and are designated A spermatogonia that appear as linear syncytia of 2, 4, 8, or 16 cells (de Rooij 1973, de Rooij *et al.* 1989). These undifferentiated spermatogonia are unique cells that are capable of forming spermatozoa once entering the ‘differentiation’ pathway as a result of the direct action of RA (Zhou *et al.* 2008a). Only after the action of RA, they undergo five carefully timed mitotic events to form A1, A2, A3, A4, intermediate, and B spermatogonia ultimately to form preleptotene spermatocytes that are

generally considered to be the entry point into meiosis (de Rooij 1973, de Rooij *et al.* 1989). Spermatogonial differentiation differs in detail between humans and mice, but the necessity for some mitotic amplification and the commitment to differentiation as a result of the action of RA is likely to be similar (Griswold *et al.* 2012, Griswold 2016). Once committed to meiosis, the steps and cell types of spermatogenesis are conserved between mice and humans (Sharma 2011). After meiosis, four haploid gametes, termed ‘round spermatids’, result from the meiotic division of every spermatocyte. Each round spermatid then undergoes unique changes in its cellular morphology and gene expression (spermiogenesis) to form a spermatozoon.

Because of the requirement for the interaction of many cell types, spermatogenesis cannot be successfully maintained by manipulating germ cells *in vitro* (Griswold 2012). In the testis, spermatogenesis takes place in the seminiferous tubules that consist of Sertoli cells and germ cells surrounded by peritubular cells providing structural support. Sertoli cells are epithelial cells that extend from the basement membrane of the tubules to the lumen and have membrane to membrane contact with each developmental stage of germ cells (Griswold *et al.* 1988, Griswold 1995). The Sertoli cells also have receptors for and respond to follicle-stimulating hormone (FSH) and testosterone that are the classical hormonal regulators of spermatogenesis (Sharpe 1987, 1989). Among their functions, Sertoli cells are crucial for stimulating the spermatogonia to enter the differentiation pathway, maintaining and degrading the tight junctions between adjacent Sertoli cells, and actively facilitating the release of spermatozoa into the lumen of the tubule. Each of these processes has been shown to be highly responsive to RA synthesized initially by Sertoli cells (Griswold *et al.* 1989). The main focus of this review is RA regulation of spermatogonial differentiation and how the action of vitamin A on this process and on the organization of spermatogenesis in time and space is critical for normal continual sperm production.

Organization of the testis

The seminiferous epithelium of mammals is highly organized (Sharma 2011). The goal of this organization is to assure a constant supply of millions of spermatozoa. In the mouse, it takes about 35 days to produce immature spermatozoa after the stimulation of undifferentiated spermatogonia to enter into differentiation. This stimulation is the result of the action of RA and will be described in detail below. Once RA acts on undifferentiated

spermatogonia, the time required to form spermatozoa is species-specific but can range from 30 to over 70 days. In order to assure a constant supply of sperm, the action of RA to stimulate undifferentiated spermatogonia is staggered and progressive along the seminiferous tubules in the form of RA pulses that occur every 8.6 days (Hogarth & Griswold 2010, 2013, Hogarth *et al.* 2015a). As the differentiating spermatogonia progress to sperm over 35 days, they move toward the lumen and overlap the germ cells developing from previous pulses. Well-defined groups of germ cells appear along the tubule sequentially or at a single points in the tubule at different times. These defined groups of germ cells, termed ‘stages’ (also known as cell associations), are morphologically distinct and can be identified in histological testis tubule cross-sections such as the one pictured in Fig. 1A (Clermont 1972). A full description of different mammalian staging schemes is beyond the scope of this review; however, more detail can be found in Russell *et al.* (1990). Note that germ cell development is a continuum and the recognized stages are not static. Stages are often depicted in charts showing the types of associations that can occur for a particular species. For example, there are 12 identifiable stages in the mouse and 14 in the rat, and each stage is represented by a specific set of germ cells being present at a single point on the tubule at the same time (Leblond & Clermont 1952). If we were able to observe germ cell differentiation over time at a single point along the tubule, we would see the appearance of a defined group of germ cell associations followed by several others and then the reappearance of the original set of associations, constituting the cycle of the seminiferous epithelium. Every 8.6 days in the mouse, at any given point

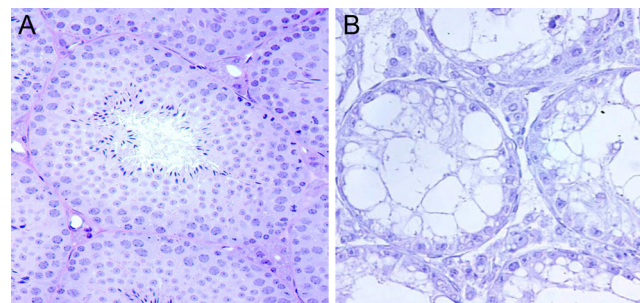


Figure 1

Histology of the seminiferous epithelium. Panel A shows the cross-section of a seminiferous tubule from a 60-day old mouse. This tubule contains a full complement of developing germ cells including spermatogonia, spermatocytes and spermatids. Panel B shows a similar section from a mouse that was given a dietary retinoic acid deficiency. The tubules contain only Sertoli cells and a few undifferentiated spermatogonia. The retinoic acid is necessary for undifferentiated spermatogonia to enter the differentiation pathway.

along a tubule, spermatogonia will differentiate at the base of the tubule and spermatozoa will be released into the lumen. In the mouse, these two coordinated processes occur during stage VIII and both are regulated by the presence of a RA pulse (Griswold 2016).

Retinoic acid and the testis

FSH and androgens have long been known to be linked with spermatogenesis, but the actions of RA on sperm production are essential and less well-characterized. Retinol is transported from liver storage to the testis via serum retinol-binding protein (RBP4). In the testis, the retinol becomes intracellular when RBP4 interacts with Stra6 that is initially expressed on the surface of Sertoli cells and later on premeiotic spermatocytes (Isken *et al.* 2008, Berry *et al.* 2013). The intracellular retinol-binding protein (RBP1) is expressed in both Sertoli cells and premeiotic germ cells, while the cellular RA-binding protein (CRABP) is highly expressed in premeiotic germ cells (Huggenvik & Griswold 1981, Ong *et al.* 1987). The testis cells express the known retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Griswold *et al.* 1989, Eskild *et al.* 1991, van Pelt *et al.* 1992, Akmal *et al.* 1997). The isoforms of RAR and RXR are found in both early germ cells and Sertoli cells, but it is clear that RA mediates its effects primarily via the action of RAR α that is highly expressed in Sertoli cells (Vernet *et al.* 2006a,b). RAR α -knockout males are sterile, and Sertoli cell-specific RAR α knockout mice have all of the characteristics of the global knockout. These data show that Sertoli cell expression of RAR α is critical for normal spermatogenesis. RAR α and RAR γ are expressed in early germ cells, but cell-specific gene deletion experiments showed that only RAR γ is required in germ cells for normal spermatogenesis (Gely-Pernot *et al.* 2012). The deletion of RAR β in male mice has no effect on spermatogenesis (Vernet *et al.* 2006a,b, Gely-Pernot *et al.* 2015, Mark *et al.* 2015). In addition, pharmacological approaches have provided additional insight into the requirement for RARs. When the pan-RA receptor antagonist BMS-189453 was used, spermatogenesis was disrupted and the testicular histology of the treated mice resembled that of vitamin A deficient or RAR α knockout mice (Chung *et al.* 2011, 2013, 2016, Noman *et al.* 2020).

The proposed model for the action of RA on spermatogenesis starts with the synthesis of RA by Sertoli cells (Griswold 2016). Retinol can be converted to retinal by retinol dehydrogenase 10 (RDH10) and to RA primarily by retinal dehydrogenase ALDH1A1 in Sertoli cells (Tong *et al.* 2013, Arnold *et al.* 2015). Low levels of ALDH1A2 are also

found in Sertoli cells (Fig. 2). The source of RA is clearly the Sertoli cells in the initiation of spermatogenesis (Teletin *et al.* 2017, Topping & Griswold 2022). The Sertoli cell synthesizes RA that then activates gene expression in undifferentiated spermatogonia or progenitor cells to undergo the transition to differentiating spermatogonia. In mice, this activation event irreversibly initiates the 35-day timed differentiation process including meiosis and ultimately ends up in the formation of spermatozoa. Exactly how the RA synthesized in Sertoli cells is able to transport to and activate RAR receptors in germ cells is unknown. Once the differentiating spermatogonia transition to spermatocytes, they acquire the capability to synthesize RA and maintain spermatogenesis independent of RA from Sertoli cells (Raverdeau *et al.* 2012, Teletin *et al.* 2019).

The action of RA on undifferentiated spermatogonia results in the induction of key genes such as *Stra8* and *Kit* both necessary for the completion of meiosis and the decreased transcription of genes such as *Pou5f1*, *Nanos2*, *Lin28a* and *Zbtb16* that characterize the undifferentiated spermatogonia (Gewiss *et al.* 2021b). The induction of *Stra8* as a result of RA is dramatic and has been used as a marker for the onset of differentiation (Gewiss *et al.* 2020). *Stra8* apparently regulates a broad gene expression program and binds the promoter of thousands of genes (Kojima *et al.* 2019). The *Stra8* gene is indispensable for meiosis, and its deletion results in a block of spermatogenesis at the preleptotene/zygotene/pachytene stages (Anderson *et al.* 2008, Mark *et al.* 2008). It has been proposed that RA acts at the levels of spermatogonial differentiation and meiotic initiation that both occur at stage VIII in the mouse. However, gene deletions that eliminated the ability to synthesize RA in both Sertoli cells and germ cells showed that while RA is required for spermatogonial differentiation, it is not required for the initiation of meiosis. This was shown in experiments where all three *Aldh1a* genes were deleted in both germ cells and Sertoli cells. When the mice were injected with RA, the spermatogonia entered the differentiation pathway and proceeded through spermatogenesis even though the injected RA was gone (Teletin *et al.* 2019). It is important to note that germ cells will enter meiosis but not complete it in the absence of *Stra8*, but undifferentiated spermatogonia never enter into differentiation in the absence of RA.

The retinoic acid pulse

Using organ culture of mouse testes and the expression of *Stra8* as a marker for RA activity, it was shown that bis-(dichloroacetyl)-diamines (BDADs) or more specifically,

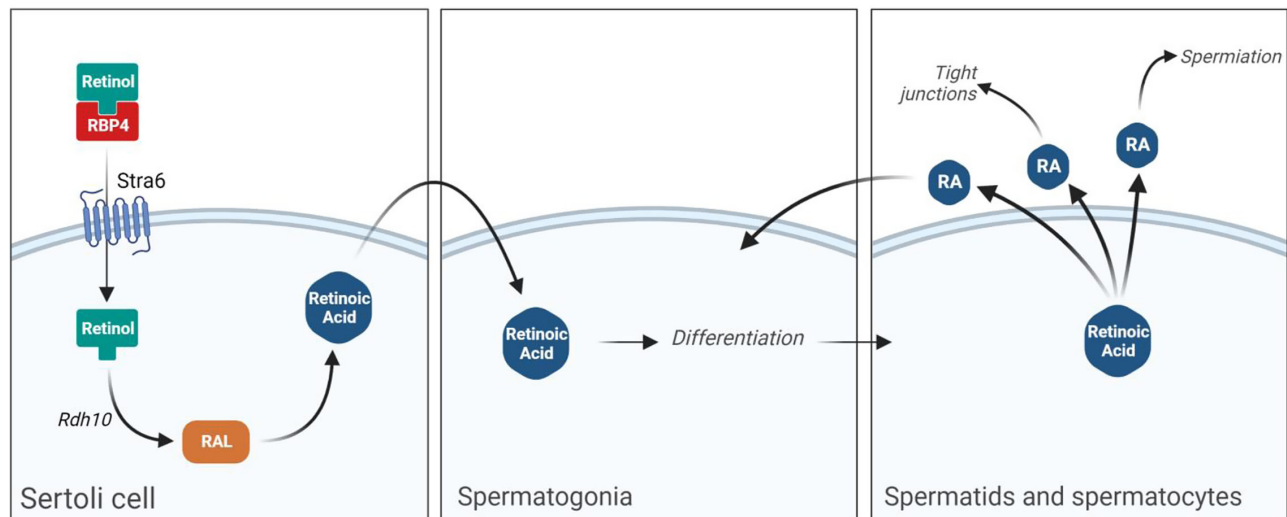


Figure 2

Model for the action of retinoic acid on spermatogenesis. Initially, retinol binds to serum retinol-binding protein (RBP4) and enters the Sertoli cells that express Stra6. Retinol inside the Sertoli cells can be bound by intracellular retinol-binding protein (RBP1) and is metabolized to retinal by RDH10. Retinal is metabolized to retinoic acid by the ALDH1A enzymes. ALDH1A1 is the main retinal dehydrogenase in Sertoli cells, but ALDH1A2 is also present. The retinoic acid is then transported by unknown mechanisms to undifferentiated spermatogonia where it can be bound to cellular retinoic acid-binding protein (CRABP) and alter the transcription of many genes-activating transcription of genes such as Kit and Stra8 and shutting down transcription of pluripotency genes such as *Zbtb16* and *Pou5f1* (Gewiss *et al.* 2021a). Once into the differentiation pathway, the formation of spermatocytes and possibly spermatids can synthesize retinoic acid via RDH10 and ALDH1A2 to drive spermiation, tight junction formation, and feedback to undifferentiated spermatogonia. Once these advanced germ cells are present, the synthesis of retinoic acid by the Sertoli cells or the germ cells can drive continuous spermatogenesis. Sertoli cells express RAR α receptors, while germ cells express both RAR α and RAR γ .

the BDAD WIN 18,446 inhibited the biosynthesis of RA from retinol in the neonatal and adult tissue (Hogarth *et al.* 2011a,b). The inhibition was shown to result from the inhibitory action of WIN 18,446 on the family of Aldh1a enzymes in a tissue-specific manner. WIN 18,446 caused only a 50% decrease in liver RA, but testicular RA decreased by over 90% (Arnold *et al.* 2015). When given to neonatal mice, WIN 18,446 blocked the progression of A undifferentiated spermatogonia to enter the differentiation pathway. The undifferentiated spermatogonia accumulated in the seminiferous tubules but could not progress (Zhou *et al.* 2008b). When the WIN 18,446 treated mice were injected with RA, the accumulated undifferentiating spermatogonia all entered the differentiation pathway simultaneously resulting in a testis with synchronous spermatogenesis (Hogarth *et al.* 2013, Evans *et al.* 2014). This synchronous germ cell development was maintained in the adult where the testis histology showed the presence of a few related stages or sometimes a single stage of the 12 stages of the cycle of the seminiferous epithelium. This treatment was used to obtain testes of adult mice synchronized around each of the 12 stages of the cycle (Hogarth *et al.* 2015a). When the level of RA was determined in each of these testes, it was shown that there was a peak amount of RA, presumably from synthesis, associated with stages VIII

to IX of the cycle. This peak or pulse corresponded to the point in the cycle where the A spermatogonia entered the differentiation pathway, the preleptotene spermatocytes transitioned the tight junctions and spermiation of elongated spermatids occurred. So, it is clear that the key to the organization of spermatogenesis that maintains continuous sperm production are these peaks of RA synthesis that occur at regular intervals (stages VIII and IX) along the tubules that allow the differentiation process of germ cells to begin. The distance along the tubules from a given stage to the next repeat of that stage is called a 'spermatogenic wave'. In an experiment reconstructing the seminiferous tubules in a mouse from serial sections, Nakata found 76 waves in a single testis or there were 76 RA pulses along the tubules at any one point in time (Nakata *et al.* 2021). As stages VIII and IX become stages IX and X and so on, the pulses progress along the tubule. In a synchronized testis, there is only one RA pulse comprising the whole testis while it is at stages VIII to IX. The use of WIN 18,446 has been used to obtain or characterize highly purified germ cells of a given stage (Chen *et al.* 2018, Romer *et al.* 2018). In normal unsynchronized testes, many (all) germ cell types are present at any given point in time, but in the synchronized testis, only the germ cells of one or a few stages of the cycle will be present.

The mechanism by which the RA pulse is generated and maintained along the tubule is still unknown. RDH10 appears to be the rate-limiting enzyme in the synthesis of RA (Napoli 1996). In Sertoli/germ cell RDH10 gene deletions, the transition of spermatogonia into the differentiation pathway is blocked (Tong *et al.* 2013). Transcriptome analysis of testes synchronized to different stages of the cycle has shown that RDH10 is induced at stages VIII and IX, is highly induced by exogenous RA and is likely to be the key enzyme in the generation of the pulse (Evans *et al.* 2014, Hogarth *et al.* 2015a). While germ cells can participate in the generation of the pulse of RA as described above, in WT mice or in the initiation of spermatogenesis, the Sertoli cells are likely the primary source. Once the stage progresses to X, XI and continues, the pulse of RA is degraded by the CYP26B1 enzyme. Gene deletion of CYP26B1 activity within both germ and Sertoli cells resulted in severe male subfertility, with a loss of advanced germ cells from the seminiferous epithelium (Hogarth *et al.* 2015b). So, CYP26B1 activity within either Sertoli or germ cells is essential for the normal progression of spermatogenesis and its loss can result in reduced male fertility.

Initially, the attention concerning the action of RA was focused on germ cells because it was clear that the RA-induced activation of genes was necessary for spermatogonial differentiation. More recently, the transcriptional profile of Sertoli cells in different stages of the cycle was examined (Gewiss *et al.* 2021a). Two approaches were used. First, the Sertoli cells from WT testes were labeled with RFP (red fluorescent protein), dissociated, FACS (fluorescence-activated cell sorting) sorted and subjected to scRNAseq (single-cell RNA sequencing). Secondly, the WIN 18,446 was used to synchronize the testes of mice with RFP-labeled Sertoli cells. Testes representing different stages of the cycle of the seminiferous epithelium were dissociated, the stage-specific Sertoli cells were isolated by FACS and the transcriptome was determined by RNAseq. Two major clusters of Sertoli cells were found as defined by their transcriptomes. One was associated with the location of the RA peak in stages VII to IX of the seminiferous epithelium and one cluster defined all other stages. The major number of differentially expressed genes were also found to be associated with the RA peak. *Stra6*, *RDH10* and *RBP1* were among the genes that were more highly expressed at these stages. Together, these data showed a cyclical response in gene expression corresponding to the stages associated with the RA pulse and those that were not associated with it. This study also examines this cyclical gene expression in the testes of *NANOS2* knockout mice where there were no germ cells

but normal-appearing Sertoli cells. It was found that the interaction of germ cells and Sertoli cells was needed for cyclic gene expression and to generate the RA pulse. The nature of this interaction remains unknown.

Retinoic acid in human testis

The mechanisms described above have all been investigated using the mouse as a model organism. Some aspects of the requirement for RA are found throughout all chordates and the organization of spermatogenesis so that continuous sperm production is found throughout mammals. The enzymes involved in the synthesis of and signaling by RA are found in the human testis, but there is little information available regarding the effect of RA on human male germ cells (Childs *et al.* 2011, Arnold *et al.* 2015). Human Sertoli cells express the enzymes of vitamin A metabolism as seen in the mouse, and *STRA8* is expressed in both the human embryonic ovary and the postnatal testis (Griswold *et al.* 2012). Clearly, there are large gaps in our knowledge with respect to RA regulation of human spermatogenesis; however, the high level of cell-type conservation in the testis between humans and rodents implies that vitamin A could very well be critical in humans for spermatogonial differentiation and for the cycle of the seminiferous epithelium. The effect of WIN 18,446 on spermatogenesis was observed over 50 years ago in humans before the mechanism of action on *Aldh1a* enzymes was determined (Heller *et al.* 1961). In human testis samples, *ALDH1A1* was found in Sertoli cells, while only *ALDH1A2* was found in spermatogonia, spermatids and spermatocytes similar to the results from rodents (Arnold *et al.* 2015). The RA synthetic pathway has been proposed both as a possible origin of some infertility and as a potential male contraceptive target (Hogarth *et al.* 2011a). In pilot studies, isotretinoin therapy was shown to improve sperm production in some men with oligoasthenozoospermia and has been used for treatment of non-obstructive azoospermia (Amory *et al.* 2017, 2021). WIN 18,446 is an effective contraceptive, but side effects such as susceptibility to alcohol do not make use feasible in humans. Several research groups are working on developing non-hormonal approaches to male contraception based on the blockade of RA function or biosynthesis including inhibition of RARs and *ALDH1A* enzymes (Amory 2020).

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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