



Driving asynchronous spermatogenesis: is retinoic acid the answer?

C.A. Hogarth, M.D. Griswold¹

School of Molecular Biosciences and the Center for Reproductive Biology, Washington State University, Pullman, WA, USA.

Abstract

Spermatogenesis in adult mammals is highly organized, with the goal being the uninterrupted production of sperm. How continual, asynchronous sperm production is initiated and maintained in the mammalian testis is still under investigation but retinoic acid (RA) likely plays a key role. Investigations utilizing vitamin A deficient rodents, transgenic mouse models, whole testis and isolated germ cell culture and inhibitors of vitamin A metabolism indicate that RA is required to drive spermatogonial differentiation and the release of spermatids from the seminiferous epithelium. These studies, and the fact that spermatogonial differentiation and spermatid release happen simultaneously in the adult testis, have led to the hypothesis that RA generates both the cycle of the seminiferous epithelium and the spermatogenic wave.

Keywords: germ cells, retinoic acid, spermatogenic cycle, testis.

Introduction

One of the features central to reproduction in male mammals is their ability to produce sperm continuously over a long period of time; in humans, sperm production begins at puberty and normally continues throughout a man's lifetime. It takes many weeks for a single spermatogonial stem cell to become a functional sperm yet it has been estimated that the human testis produces 1000 sperm with each heartbeat or about 37 billion sperm per year (Wade, 2004). Spermatogenesis is an incredibly complex and tightly regulated process that involves three major fundamental biological events: the renewal and mitotic production of spermatogonia; the recombination and segregation of homologous chromosomes into daughter haploid cells during meiosis; and the unique morphological and nuclear changes, collectively known as spermiogenesis, which occur to transform spermatids to spermatozoa (Fig. 1). The active metabolite of vitamin A, retinoic acid (RA), is believed to be important for all three of these processes to occur, but the regulation of RA synthesis and the downstream effects of RA signaling within the testis are only just beginning to be understood.

Mammalian spermatogenesis takes place within the seminiferous epithelium inside testis tubules. The structural integrity of this epithelium is maintained

by the developing germ cells in cell-to-cell contact with Sertoli cells, somatic cells that support spermatogenesis by sending and receiving signals between the germ cells and surrounding interstitium, and by the peritubular myoid cells (PTMs), which surround the epithelium to form seminiferous tubules. Within the interstitial space between tubules are the Leydig cells, which are responsible for the production of testosterone, and other supporting somatic cells including macrophages, blood cells, lymphatic vessels and nerves. Some of the functions of the seminiferous epithelium are directed by the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which are required for normal spermatogenesis. While LH stimulates testosterone biosynthesis, FSH maintains the function of the Sertoli cells, which nurture and promote germ cell differentiation.

Given that access to human testicular tissue for research purposes is extremely limited, our current understanding of mammalian spermatogenesis, and of vitamin A function in germ cell development, has mostly been generated via studies of rodent testis tissue. In rodents, there are two populations of spermatogonia; 1) the undifferentiated Type A spermatogonia (Asingle, Apaired, Aaligned) and 2) the differentiated spermatogonia (A₁, A₂, A₃, A₄, Intermediate and B). Undifferentiated spermatogonia divide mitotically to either repopulate the testis with spermatogonial stem cells or to provide progenitor cells that can be triggered to commit to undergo differentiation and meiosis (Herms *et al.*, 2010a). Once spermatogonia are triggered to differentiate, a process known as the A to A1 transition, they begin a series of five mitotic divisions and irreversible differentiation that result in the conversion of these cells to preleptotene spermatocytes and meiotic initiation. The spermatocytes then undergo meiosis, a process which generates genetic diversity through homologous recombination and allows proper chromosome segregation to produce haploid germ cells. In the mouse, meiotic prophase is lengthy (approximately 2 weeks) and cells are divided into different populations based on their chromatin morphology (preleptotene, leptotene, zygotene, pachytene, and diplotene; Kerr, 2006; Herms *et al.*, 2010a). The result of meiosis in the testis is the production of four round haploid spermatids from each diploid germ cell, which then undergo spermiogenesis to first form elongating spermatids and finally spermatozoa (Herms *et al.*, 2010b).

¹Corresponding author: mgriswold@vetmed.wsu.edu

Phone: +1(509)335-6281; Fax: +1(509)335-4159

Received: May 31, 2012

Accepted: September 6, 2012

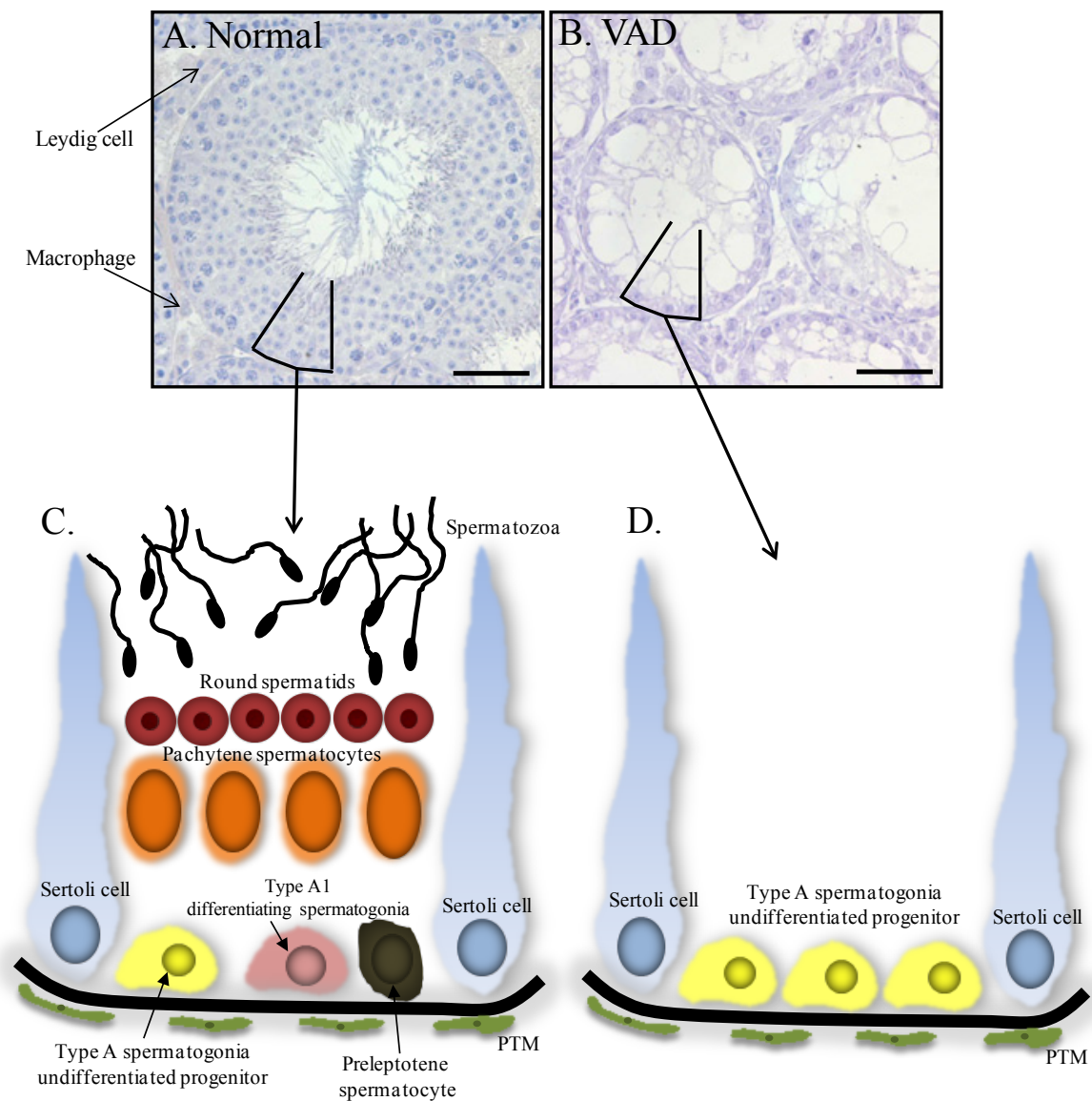


Figure 1. The normal and VAD adult mouse testis. Vitamin A deficiency results in a block during spermatogonial differentiation. Histological cross-section of (A) a normal mouse testis tubule and (B) vitamin A-deficient (VAD) tubule, stained with Harris Haematoxylin to visualize chromatin. (C) The arrangement of germ and Sertoli cells within the normal seminiferous epithelium is shown. Sertoli cells (blue) maintain contact with each germ cell type throughout their development. As spermatogenesis proceeds, the more differentiated germ cells move towards the lumen of the tubule where the spermatozoa are released. The peritubular myoid cells (PTMs; green) form the architecture of the seminiferous epithelium and also aid in moving spermatozoa through the testis tubules to be released into the male reproductive tract. (D) In the VAD mouse testis, the A to A1 transition is blocked and all differentiated germ cells are lost from the testis. The result is seminiferous tubules which contain only undifferentiated spermatogonia and Sertoli cells. Scale bars represent 50 μ m. Adapted from Hogarth *et al.* (2011a).

RA regulation of spermatogenesis

It has been known since 1925 that vitamin A is essential for normal sperm production in mammals. Wolbach and Howe first reported the early cessation of spermatogenesis in rats fed a vitamin A-deficient (VAD) diet (Wolbach and Howe, 1925) and more recent studies have demonstrated the progressive loss of all

differentiating germ cells from VAD rodent testes, leaving only Sertoli cells and undifferentiated spermatogonia within the seminiferous epithelium (Mitrandon *et al.*, 1979; Unni *et al.*, 1983; Griswold *et al.*, 1989; van Pelt and de Rooij, 1990; McLean *et al.*, 2002). In mammals, vitamin A is usually transported within the serum as retinol bound to retinoid binding proteins and conversion of this retinoid for either



storage, as retinyl esters, or immediate use as RA, takes place in target tissues (Livera *et al.*, 2002). Retinol is taken up by cells via the membrane-bound receptor STRA6 and once inside, the conversion of retinol to RA is controlled by two sequential oxidative enzymatic steps catalyzed by the retinol or alcohol dehydrogenases and retinaldehyde dehydrogenases (Theodosiou *et al.*, 2010; Fig. 2). Within cells, RA interacts with heterodimers of the RA receptors (RARs) and the retinoid X receptors (RXRs) and binds RA response elements (RAREs) in target genes, recruiting co-repressors or co-activators to either inhibit or induce transcription. The cytochrome P450 enzymes, CYP26A1, CYP26B1 and CYP26C1, degrade RA once it has exerted its effects on gene expression and so a precise balance exists between the production and degradation of RA (for a review see Duester, 2008). Our current understanding of how RA regulates spermatogenesis has been shaped by studying the cell types that harbor vitamin A metabolizing and signaling machinery and examining how germ and Sertoli cells respond to excess or deficient levels of RA.

RA regulation of spermatogonial differentiation and meiotic initiation

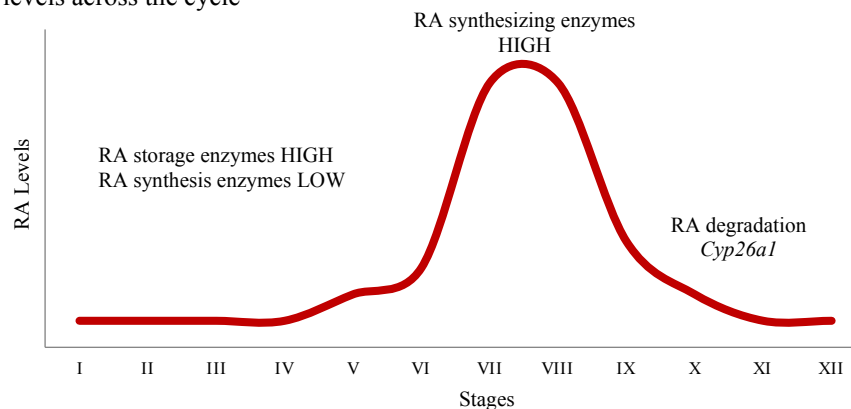
In mice, RA is absolutely required for spermatogonia to complete the A to A1 transition. As mentioned above, when adult male mice are made VAD, all differentiated germ cells are lost from the seminiferous epithelium, and only type A spermatogonia and Sertoli cells remain (Bowles and Koopman, 2007; Hogarth and Griswold, 2010; Fig. 1). When the vitamin A is replaced, either in the form of retinol or RA, spermatogonial differentiation is triggered in these testes. This indicates that removing RA blocks the ability of undifferentiated spermatogonia to differentiate. Studies in juvenile animals also support this conclusion, as dietary depletion of vitamin A results in the accumulation of undifferentiated spermatogonia and meiotic failure in young male mice null for the retinoid storage enzyme lecithin:retinol acyltransferase (*Lrat*; Li *et al.*, 2011). Recent evidence generated from RA treatment of short term cultures of neonatal testes and isolated undifferentiated spermatogonia showed an upregulation in the expression of *Kit* (Pellegrini *et al.*, 2008; Zhou *et al.*, 2008a), a marker of differentiating spermatogonia, and increased the number of cells containing nuclei reminiscent of leptotene and zygotene spermatocytes (Pellegrini *et al.*, 2008). Taken together, these studies suggest that RA can drive spermatogonia to differentiate and initiate the process of meiosis both during the first wave of spermatogenesis and in the adult testis.

The classic marker of RA activity in the testis, and at meiotic entry in the ovary, is *Stra8*. Transcriptome analysis of embryonic ovaries and postnatal testes from mice revealed that *Stra8* is most highly expressed at embryonic day 14.5 and 10 days postpartum (dpp), respectively, the timepoints

corresponding to meiotic initiation in females and males (Shima *et al.*, 2004; Small *et al.*, 2005). *Stra8* was first identified as being a direct target of RA signaling in P19 embryonic carcinoma cells (Oulad-Abdelghani *et al.*, 1996) and RA has been shown to be necessary for the induction of *Stra8* in both sexes (Koubova *et al.*, 2006), although RA action in the embryonic ovary has recently been questioned (Kumar *et al.*, 2011). Cultured neonatal testes or isolated undifferentiated spermatogonia have provided evidence to indicate that *Stra8* is a direct target of RA in spermatogonia (Zhou *et al.*, 2008a; Hogarth *et al.*, 2011b). In normal mouse testes, STRA8 protein appears to be present in subpopulations of differentiating spermatogonia and preleptotene and leptotene spermatocytes in a stage-specific manner (Zhou *et al.*, 2008b; Hogarth *et al.*, unpublished observations; Fig. 2), with the highest levels detected in stages VII and VIII of the seminiferous cycle, precisely when the A to A1 transition is taking place. Injections of either neonatal or adult vitamin A sufficient male mice with exogenous RA can also drive the expression of STRA8 in undifferentiated spermatogonia in a stage-specific manner (Snyder *et al.*, 2011; Hogarth *et al.*, unpublished observations), indicating that there may be a particular subpopulation of undifferentiated spermatogonia that are ready to respond to RA.

Taken together, these data suggest that STRA8, like RA, should be essential for the A to A1 transition, yet studies of the *Stra8*-null mouse line indicate that the story is not quite so simple. *Stra8*-deficient mice, on a purebred C57BL/6 background, are infertile due to a meiotic defect, with preleptotene cells accumulating in both the testes and ovaries (Baltus *et al.*, 2006; Anderson *et al.*, 2008). However, the background strain and animal age both significantly affect the null male phenotype. Animals on a mixed background show a more severe and leaky phenotype, with the blockage occurring anywhere from the A to A1 transition to during meiosis and as the purebred null animals age, undifferentiated spermatogonia begin to accumulate, indicating that the A to A1 transition is also susceptible to the loss of STRA8 over time (D. Page, personal communication). If the sole function of RA is to drive *Stra8* expression, then we would expect to observe a complete block during the A to A1 transition in *Stra8*-null males from the outset. These knockout mouse data indicate that *Stra8* is not initially essential for the A to A1 transition, but that STRA8-deficient preleptotene spermatocytes are incapable of progressing through meiosis and overtime, or on varying genetic backgrounds, spermatogonial differentiation does require STRA8. To date, the function of STRA8 has remained elusive, with only one study demonstrating that it can act as a transcription factor in cultured cells (Tedesco *et al.*, 2009). STRA8 does contain a putative DNA binding domain but further *in vivo* functional studies and array analyses of *Stra8*-deficient testes will be required before we can conclusively label STRA8 as a transcription factor.

A. Predicted RA levels across the cycle



B. Stages VII/VIII Model

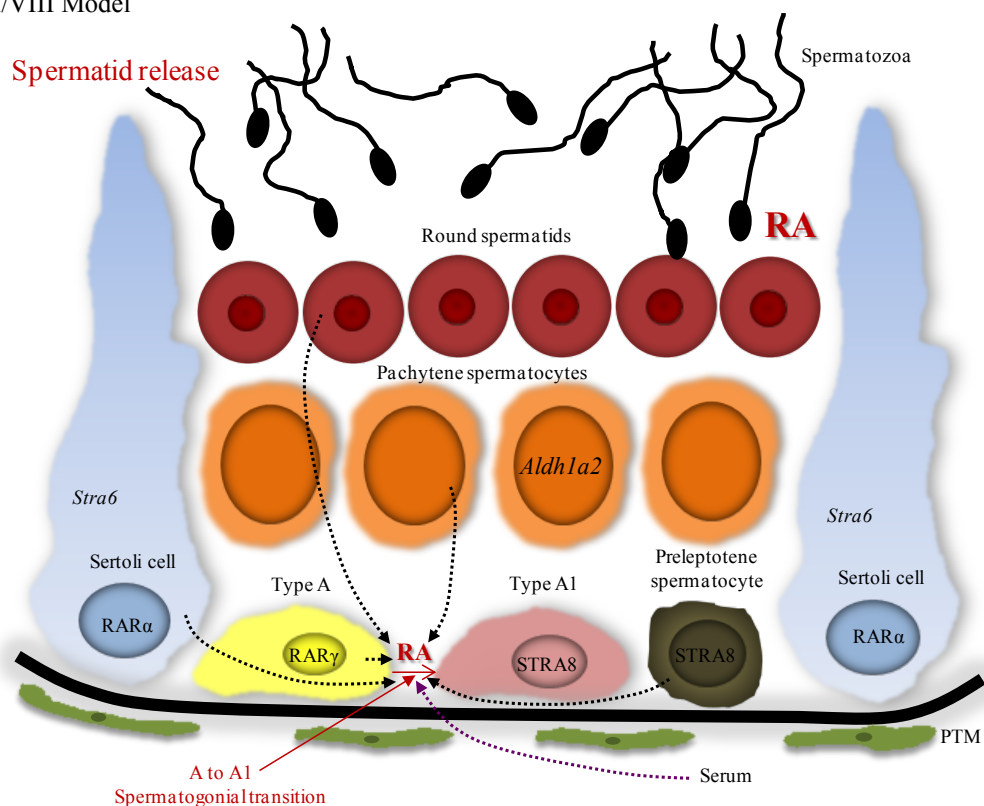


Figure 2. RA regulation of the cycle of the seminiferous epithelium. There is a body of evidence, which is continuing to build, that supports the hypothesis that RA drives the cycle of the seminiferous epithelium. Preliminary data generated from measuring RA levels and expression of retinoid storage, synthesis and degradation enzymes predicts if RA levels could be graphed as a function of the Stages of the cycle of the seminiferous epithelium (A), peak levels would be present at Stages VII and VIII. The detection of *Cy26a1* in Stages VIII through XII suggests that this cytochrome p450 enzyme is responsible for degrading RA after the A to A1 transition and spermatid release has been triggered. B) Schematic representing Stages VII and VIII of the seminiferous epithelium, when two processes relying on RA, the A to A1 spermatogonial transition and spermatid release, take place. This model predicts that the production of RA either by Sertoli cells (blue), the differentiating germ cells or possibly the undifferentiated spermatogonia (yellow) (dotted black arrows) drives the A to A1 transition and leads to the expression of STRA8 in the differentiating spermatogonia (pink). STRA8 is also present in the preleptotene and leptotene spermatocytes (black) but whether these cells produce STRA8 protein in response to RA has yet to be confirmed. Sertoli cells are believed to be able to take up RA through the surface receptor, *Stra6*. The detection of *Aldh1a2* transcripts in pachytene spermatocytes (orange) suggests that these cells are a possible source of RA for both the A to A1 transition and spermatid release. RA is believed to signal through *RARα* in the Sertoli cells and *RARγ* in the spermatogonia. The serum could also be a direct source of RA for the A to A1 transition and spermatid release (purple dotted arrow).



While *Stra8* is an important RA target gene that regulates meiotic progression in rodents, there must be other RA-responsive targets which are critical for the A to A1 spermatogonial transition. Array studies on either whole testes or isolated germ cells treated with RA have been performed but other than *Stra8*, a standout germ cell-specific target has yet to be identified (Pellegrini *et al.*, 2008; Zhou *et al.*, 2008a; Hogarth *et al.*, 2011b). This is partly because we have yet to determine whether RA acts on Sertoli cells, germ cells, or both to drive the A to A1 transition. Expression studies have demonstrated that both spermatogonia and Sertoli cells contain the vitamin A metabolizing enzymes necessary to convert retinol to RA (Deltour *et al.*, 1997; Lopez-Fernandez and del Mazo, 1997; Zhai *et al.*, 1997, 2001; Kasus-Jacobi *et al.*, 2005; Vernet *et al.*, 2006; Hu *et al.*, 2007). Both cell types also express the receptors necessary for RA signaling. Studies of the two receptor families, the RARs and RXRs, have shown that many isoforms of these receptors are found in various testis cell types at different developmental stages (Akmal *et al.*, 1997; Dufour and Kim, 1999; Vernet *et al.*, 2006) suggesting that most testicular cells can respond to RA. Analysis of animals displaying global and cell-specific deficiencies of the RA receptors have led to the current model suggesting that RA mediates its effects primarily via the action of RAR α in Sertoli cells and via the action of RAR γ in early germ cells (Fig. 2). *Rara*-knockout males are sterile, and transplantation experiments demonstrated that *Rara*-null germline stem cells could colonize a germ cell-depleted testis and progress through spermatogenesis normally (Doyle *et al.*, 2007). This suggests that Sertoli cell expression of RAR α is critical for normal spermatogenesis. Initial analyses of the *Rar γ* -knockout males suggested that their sterility phenotype was the result of losing secretory functions of the male genital tract (Lohnes *et al.*, 1993) but recent examination of *Rar γ* -deficient testes revealed that this receptor is essential for the A to A1 transition in the adult mouse. Gely-Pernot *et al.* (2012) found RAR γ to be present on undifferentiated A_{aligned} spermatogonia and on differentiating A1 spermatogonia, and the testes of knockout animals displayed a VAD-like phenotype by 15 weeks of age when fed vitamin A sufficient diets. Interestingly, no defects in the A to A1 transition were observed in the testes of animals aged 6 weeks or younger, indicating that while RAR γ appears to be essential for spermatogonial differentiation in the adult mouse, there are mechanisms in place in the juvenile testis to overcome the loss of this receptor. Continued investigation of how and where RA is produced in the testis and which receptors are responsible for transducing the RA signal will be critical to determining how RA drives spermatogonial differentiation. High-throughput sequencing analyses utilizing wild-type and knockout mouse models will be important to ascertain the downstream targets of RA signaling in

spermatogonia.

RA regulation of spermatid development

Much of our understanding of how RA regulates the development of spermatids has been derived from studies of VAD and knockout mouse models and expression analyses. Three of the retinoid receptors, RAR β , RXR α , and RXR β , have been reported to be present on spermatids, cells which also contain enzymes that metabolize and store vitamin A (Vernet *et al.*, 2006; Wu *et al.*, 2008). A direct link between vitamin A and spermatid function was first reported in 1983 when Huang and Marshall observed a disruption in how and when spermatids were released from the seminiferous epithelium in young rats becoming VAD (Huang and Marshall, 1983). More recent studies have confirmed that RA signaling within the Sertoli cell is required for the normal release of spermatids and revealed it to be necessary for the proper alignment of spermatids within the seminiferous epithelium. Analysis of animals carrying targeted deletions of either *Rxr β* or *Rara* in Sertoli cells demonstrated that these receptors may act as a homodimer to coordinate normal spermatid release (Chung *et al.*, 2004, 2005; Vernet *et al.*, 2008). Close examination of elongating spermatids in the testes of *Rara*-deficient animals indicated that signaling through this receptor is important for the orientation of step8/9 spermatids within the seminiferous epithelium, leading to a temporary arrest of spermatid development at these steps (Chung *et al.*, 2004, 2005). Significant apoptosis of elongating spermatids was also observed in *Rara*-deficient testes. In addition, treatment of male mice with low doses of the pan-RAR antagonist, BMS-189453, resulted in the failure of spermatid alignment and release in a manner that closely resembled those observed for VAD rodents and the *Rara*-deficient mouse (Chung *et al.*, 2011). Collectively, these studies indicate that proper RA production and signaling is essential for normal spermiogenesis. Further investigation of how RA acts as the messenger between Sertoli cells and spermatids to drive spermatid release from the seminiferous epithelium has important implications for whether this mechanism can be exploited for the purposes of male contraception.

Does RA drive the cycle of the seminiferous epithelium?

The adult mouse testis is organized so that every 8.6 days at any given point along a testis tubule, a type A undifferentiated spermatogonia undergoes the A to A1 transition while a spermatozoa is released from the seminiferous epithelium into the tubule lumen. Continuous sperm production is achieved by coordinating spermatogenesis such that specific groups of germ cells are always in association, identified as



Stages, and appear in a reoccurring sequence along the testis tubule (Clermont, 1972). If we were able to observe germ cell differentiation over time at a single point on the tubule, we would see the appearance of a defined group of cell associations followed by several others and then the reappearance of the original set of associations, constituting the cycle of the seminiferous epithelium. However, if a mouse testis is analyzed in cross-section, we see tubules representing all 12 Stages of the mouse cycle, demonstrating that the different germ cell associations are always present among the testis as a whole. The net result of the cycle is the asynchronous, phased release of spermatozoa along the entire length of the testis tubule, continuous sperm production. In the mouse testis, the cycle takes 8.6 days and there is a growing body of evidence to suggest that this cycle is driven by RA.

Evidence to support a role for RA in the establishment of the cycle has been derived from studies of animals with testes containing only undifferentiated spermatogonia and Sertoli cells, with the most well-characterized being VAD rodents. Asynchronous sperm production is eliminated in VAD rodents, but interestingly, when retinol is given back to these animals, spermatogenesis is reinitiated by stimulating the A to A1 transition in a synchronized manner throughout the entire testis. The result is that all germ cells will mature in synchrony and rather than all 12 Stages of the cycle being present, only one or two sequential Stages can be found in all tubules across a histological cross-section of these testes (Griswold *et al.*, 1989). This synchronization is maintained for many months and results in the release of spermatozoa only every 8.6 days. Therefore vitamin A-driven synchronization of the mouse testis eliminates continual sperm production. RA-induced synchronous differentiation of spermatogonia was also observed in the testes of artificial cryptorchids, *Nanos3*-null mice and *W/W^v* mutants, all mouselines with testes containing only Sertoli cells and undifferentiated spermatogonia (Sugimoto *et al.*, 2012). In addition to synchronizing germ cell development, RA treatment of VAD rodents also appears to reset the Sertoli cell cycle. In the normal adult rodent testis, Sertoli cells exhibit cyclic changes in their function and gene expression (Elftman, 1950; Timmons *et al.*, 2002; Johnston *et al.*, 2008). These changes coincide with the different Stages, indicating that Sertoli cells work to provide the microenvironment appropriate for each set of germ cell associations. In VAD testes, normal Stage-specific patterns of Sertoli cell gene expression were observed (Sugimoto *et al.*, 2012), suggesting that Sertoli cells can cycle normally in the absence of differentiating germ cells and vitamin A. However, treatment of VAD mice with RA induced uniform Sertoli cell gene expression, indicating that the Sertoli cells had reset their cycle to match that of the associated synchronously differentiating germ cells (Sugimoto *et al.*, 2012).

While studies of VAD rodents show that RA can control the cycle of the seminiferous epithelium after it has been disturbed, evidence derived from neonatal animals suggest that this compound can induce changes in the cycle before it has even been established. When the testes of mice that had been injected with a single dose of RA at 2 dpp and then left to recover to adulthood were examined, very few Stages were present indicating that RA treatment of neonatal male mice can induce synchronous spermatogenesis in the adult testis (Snyder *et al.*, 2011). Immunohistochemical staining for STRA8 protein 24 h after treatment and the use of a transgenic mouse line, *RAREhspLacZ*, that expresses β -galactosidase in response to RA signaling suggested that all germ cells within a 2 dpp testis can differentiate in response to exogenous RA, leading to their synchronous development and loss of continual sperm production when these animals are left to recover to adulthood. Detection of STRA8 protein in wild-type neonatal mice and β -galactosidase in untreated neonatal *RAREhspLacZ* animals indicate that the germ cell response to endogenous RA in a wild-type testis is heterogeneous (Snyder *et al.*, 2010); not all tubules contain germ cells that have entered the differentiation pathway and not all germ cells within one testis tubule enter the differentiation pathway at the same time. Taken together, these studies imply that the precise production and degradation of RA within neonatal testis tubules could drive the cycle of the seminiferous epithelium and continual sperm production by triggering the A to A1 transition in a heterogeneous manner.

Gene expression studies analyzing the production of the vitamin A metabolizing enzymes in a Stage-specific manner are beginning to provide evidence to support the hypothesis that RA levels are precisely controlled within a testis tubule. There are 12 Stages of the mouse cycle of the seminiferous epithelium, with the A to A1 transition and the release of spermatozoa from the seminiferous epithelium taking place simultaneously in Stages VII and VIII. In situ hybridization studies by Vernet *et al.* (2006) and Sugimoto *et al.* (2012), imply that the genes which control RA synthesis, uptake, storage and degradation are expressed in a tightly regulated Stage-specific manner. Data derived from both studies suggest that the endogenous RA concentration is low in Stages I-VI, before the A to A1 transition/spermatozoa release takes place, as there are very low or undetectable levels of transcripts coding for RA synthesizing enzymes. *Stra6* mRNA is present in Sertoli cells, with the highest levels detected in Stages VII-IX, indicating that these cells are actively producing the protein responsible for RA uptake (Sugimoto *et al.*, 2012). *Aldh1a2* transcripts were also observed in pachytene spermatocytes in Stages VII-XII, suggesting that RA is being produced by the differentiating germ cells during these Stages (Vernet *et al.*, 2006; Sugimoto *et al.*, 2012). Preliminary measurements of retinoid levels within testis tubules of



the different Stages also imply that Stages VII-VIII contain the highest levels of endogenous RA (Hogarth *et al.*, unpublished observations). RA degradation also appears to be promoted during Stages VIII-XII as *Cyp26a1* mRNA can be detected, with the highest levels observed in Stages VIII-XI (Sugimoto *et al.*, 2012). Taken together, these expression studies imply that there is a sharp increase in endogenous RA production followed by a rapid decrease in local RA levels at Stages VII-VIII of the seminiferous epithelium, ultimately leading to RA driving the cycle of the seminiferous epithelium.

Conclusions

The body of data summarized above provides a strong case to support the hypothesis that RA both initiates and maintains the cycle of the seminiferous epithelium and is diagramed in Fig. 2. Spermatogonial differentiation and the release of spermatids into the tubule lumen, the beginning and end of spermatogenesis within the testis respectively, both rely on RA, and happen simultaneously during Stages VII/VIII of the cycle, when RA levels appear to be at their highest. RA can drive the cycle of the seminiferous epithelium to synchronize either before its establishment in the neonatal animal or after it has been perturbed, as is the case in the VAD rodent. In addition, preliminary expression studies suggest that the vitamin A metabolizing and signaling machinery is localized such that RA could be produced in a very precise manner at specific points along the testis tubule. The task for researchers now is to verify that the proteins required for the tightly regulated delivery of RA at specific points along the tubule are present, as publications to date have only reported transcript expression data, and to overcome the current technical limitations associated with accurately measuring retinoid levels in testis tissues. A highly sensitive LC/MS/MS assay for retinoid detection has been developed (Arnold *et al.*, 2012) and this should allow for progress to be made on measuring RA levels in the testis. In addition, further study of cell-specific gene knockouts of the metabolizing and signaling machinery will be important for determining how interactions between germ and Sertoli cells influence RA's effect on the cycle. Not only will continued investigations into how RA regulates spermatogenesis help to determine how male mammals continue to produce sperm, but these studies may also have implications for the treatment of infertility and the ongoing pursuit of a non-hormonal male contraceptive.

Acknowledgments

The authors would like to thank Chris Small for his critical reading of the manuscript. The research was supported by NIH Grant HD 10808 to MDG.

References

- Akmal KM, Dufour JM, Kim KH.** 1997. Retinoic acid receptor alpha gene expression in the rat testis: potential role during the prophase of meiosis and in the transition from round to elongating spermatids. *Biol Reprod*, 56:549-556.
- Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC.** 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci USA*, 105:14976-14980.
- Arnold SL, Amory JK, Walsh TJ, Isoherranen N.** 2012. A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS. *J Lipid Res*, 53:587-598.
- Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, Page DC.** 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet*, 38:1430-1434.
- Bowles J, Koopman P.** 2007. Retinoic acid, meiosis and germ cell fate in mammals. *Development*, 134:3401-3411.
- Chung SS, Sung W, Wang X, Wolgemuth DJ.** 2004. Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. *Dev Dyn*, 230:754-766.
- Chung SS, Wang X, Wolgemuth DJ.** 2005. Male sterility in mice lacking retinoic acid receptor alpha involves specific abnormalities in spermiogenesis. *Differentiation*, 73:188-198.
- Chung SS, Wang X, Roberts SS, Griffey SM, Reczek PR, Wolgemuth DJ.** 2011. Oral administration of a retinoic acid receptor antagonist reversibly inhibits spermatogenesis in mice. *Endocrinology*, 152:2492-2502.
- Clermont Y.** 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev*, 52:198-236.
- Deltour L, Haselbeck RJ, Ang HL, Duester G.** 1997. Localization of class I and class IV alcohol dehydrogenases in mouse testis and epididymis: potential retinol dehydrogenases for endogenous retinoic acid synthesis. *Biol Reprod*, 56:102-109.
- Doyle TJ, Braun KW, McLean DJ, Wright RW, Griswold MD, Kim KH.** 2007. Potential functions of retinoic acid receptor A in Sertoli cells and germ cells during spermatogenesis. *Ann NY Acad Sci*, 1120:14-130.
- Duester G.** 2008. Retinoic acid synthesis and signaling during early organogenesis. *Cell*, 134:921-931.
- Dufour JM, Kim KH.** 1999. Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. *Biol Reprod*, 61:1300-1308.
- Eltman H.** 1950. The Sertoli cell cycle in the mouse. *Anat Rec*, 106:381-392.



- Gely-Pernot A, Raverdeau M, Celebi C, Dennefeld C, Feret B, Klopfenstein M, Yoshida S, Ghyselinck NB, Mark M.** 2012. Spermatogonia differentiation requires retinoic acid receptor gamma. *Endocrinology*, 153:438-449.
- Griswold MD, Bishop PD, Kim KH, Ping R, Siiteri JE, Morales C.** 1989. Function of vitamin A in normal and synchronized seminiferous tubules. *Ann NY Acad Sci*, 564:154-172.
- Hermo L, Pelletier RM, Cyr DG, Smith CE.** 2010a. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. *Microsc Res Tech*, 73:241-278.
- Hermo L, Pelletier RM, Cyr DG, Smith CE.** 2010b. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: changes in spermatid organelles associated with development of spermatozoa. *Microsc Res Tech*, 73:279-319.
- Hogarth CA, Griswold MD.** 2010. The key role of vitamin A in spermatogenesis. *J Clin Invest*, 120:956-962.
- Hogarth CA, Amory JK, Griswold MD.** 2011a. Inhibiting vitamin A metabolism as an approach to male contraception. *Trends Endocrinol Metab*, 22:136-144.
- Hogarth CA, Evanoff R, Snyder E, Kent T, Mitchell D, Small C, Amory JK, Griswold MD.** 2011b. Suppression of Stra8 expression in the mouse gonad by WIN 18,446. *Biol Reprod*, 84:957-965.
- Hu P, Zhang M, Napoli JL.** 2007. Ontogeny of rdh9 (Crad3) expression: ablation causes changes in retinoid and steroid metabolizing enzymes, but RXR and androgen signaling seem normal. *Biochim Biophys Acta*, 1770: 694-705.
- Huang HF, Marshall GR.** 1983. Failure of spermatid release under various vitamin A states: an indication of delayed spermiation. *Biol Reprod*, 28:1163-1172.
- Johnston DS, Wright WW, Dicandoloro P, Wilson E, Kopf GS, Jelinsky SA.** 2008. Stage-specific gene expression is a fundamental characteristic of rat spermatogenic cells and Sertoli cells. *Proc Natl Acad Sci USA*, 105:8315-8320.
- Kasus-Jacobi A, Ou J, Birch DG, Locke KG, Shelton JM, Richardson JA, Murphy AJ, Valenzuela DM, Yancopoulos GD, Edwards AO.** 2005. Functional characterization of mouse RDH11 as a retinol dehydrogenase involved in dark adaptation in vivo. *J Biol Chem*, 280:20413-20420.
- Kerr JB, Loveland KL, O'Bryan MK, deKretser DM.** 2006. Cytology of the testis and intrinsic control mechanisms. In: Neill JD. (Ed.). *Physiology of Reproduction*. New York: Elsevier. pp. 827-947.
- Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC.** 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci USA*, 103:2474-2479.
- Kumar S, Chatzi C, Brade T, Cunningham TJ, Zhao X, Duester G.** 2011. Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signalling. *Nat Commun*, 2, 151.
- Li H, Palczewski K, Baehr W, Claggett-Dame M.** 2011. Vitamin A deficiency results in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis. *Biol Reprod*, 84:336-341.
- Livera G, Rouiller-Fabre V, Pairault C, Levacher C, Habert R.** 2002. Regulation and perturbation of testicular functions by vitamin A. *Reproduction*, 124:173-180.
- Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P.** 1993. Function of retinoic acid receptor gamma in the mouse. *Cell*, 73:643-658.
- Lopez-Fernandez LA, del Mazo J.** 1997. The cytosolic aldehyde dehydrogenase gene (Aldh1) is developmentally expressed in Leydig cells. *FEBS Lett*, 407:225-229.
- McLean DJ, Russell LD, Griswold MD.** 2002. Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. *Biol Reprod*, 66:1374-1379.
- Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W.** 1979. Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. *Acta Anat (Basel)*, 103:159-168.
- Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P.** 1996. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *J Cell Biol*, 135:469-477.
- Pellegrini M, Filippini D, Gori M, Barrios F, Lolicato F, Grimaldi P, Rossi P, Jannini EA, Geremia R, Dolci S.** 2008. ATRA and KL promote differentiation toward the meiotic program of male germ cells. *Cell Cycle*, 7:3878-3888.
- Shima JE, McLean DJ, McCarrey JR, Griswold MD.** 2004. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biol Reprod*, 71:319-330.
- Small CL, Shima JE, Uzumcu M, Skinner MK, Griswold MD.** 2005. Profiling gene expression during the differentiation and development of the murine embryonic gonad. *Biol Reprod*, 72:492-501.
- Snyder EM, Small C, Griswold MD.** 2010. Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. *Biol Reprod*, 83:783-790.
- Snyder EM, Davis JC, Zhou Q, Evanoff R, Griswold MD.** 2011. Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. *Biol Reprod*, 84:886-893.
- Sugimoto R, Nabeshima Y, Yoshida S.** 2012. Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mech Dev*, 128:610-624.



- Tedesco M, La Sala G, Barbagallo F, De Felici M, Farini D.** 2009. STRA8 shuttles between nucleus and cytoplasm and displays transcriptional activity. *J Biol Chem*, 284:35781-35793.
- Theodosiou M, Laudet V, Schubert M.** 2010. From carrot to clinic: an overview of the retinoic acid signaling pathway. *Cell Mol Life Sci*, 67:1423-1445.
- Timmons PM, Rigby PW, Poirier F.** 2002. The murine seminiferous epithelial cycle is pre-figured in the Sertoli cells of the embryonic testis. *Development*, 129:635-647.
- Unni E, Rao MR, Ganguly J.** 1983. Histological & ultrastructural studies on the effect of vitamin A depletion & subsequent repletion with vitamin A on germ cells & Sertoli cells in rat testis. *Indian J Exp Biol*, 21:180-192.
- van Pelt AM, de Rooij DG.** 1990. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biol Reprod*, 43:363-367.
- Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, Mark M.** 2006. Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology*, 147:96-110.
- Vernet N, Dennefeld C, Klopfenstein M, Ruiz A, Bok D, Ghyselinck NB, Mark M.** 2008. Retinoid X receptor beta (RXRB) expression in Sertoli cells controls cholesterol homeostasis and spermiation. *Reproduction*, 136:619-626.
- Wade N.** 2004. Sperm stem cells are grown outside body. *The New York Times*, New York, Nov. 4, 2004.
- Wolbach SB, Howe PR.** 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J Exp Med*, 42:753-777.
- Wu JW, Wang RY, Guo QS, Xu C.** 2008. Expression of the retinoic acid-metabolizing enzymes RALDH2 and CYP26b1 during mouse postnatal testis development. *Asian J Androl*, 10:569-576.
- Zhai Y, Higgins D, Napoli JL.** 1997. Coexpression of the mRNAs encoding retinol dehydrogenase isozymes and cellular retinol-binding protein. *J Cell Physiol*, 173:36-43.
- Zhai Y, Sperkova Z, Napoli JL.** 2001. Cellular expression of retinal dehydrogenase types 1 and 2: effects of vitamin A status on testis mRNA. *J Cell Physiol*, 186:220-232.
- Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, Griswold MD.** 2008a. Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. *Biol Reprod*, 78:537-545.
- Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess R, Small C, Griswold MD.** 2008b. Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. *Biol Reprod*, 79:35-42.
-