Retinoic Acid Signaling in Spermatogenesis and Male (In)Fertility

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Key points:
Retinoids and RA are essential players in biological signaling pathways.
Retinoic acid (RA) has a vital role in spermatogenesis and male fertility.
RA is involved in sperm metabolism and oxidative stress.
Disturbed RA signaling contributes to human male infertility.

ABSTRACT

Spermatogenesis is a very complex, highly organised and timely regulated event, in which spermatozoa originated from undifferentiated spermatogonia that are recruited into the differentiation pathway and meiosis. The spermatogonia entrance into the differentiation pathway is temporally controlled, which in mammals occurs in a progressive non-uniform manner along the tubule at space intervals, allowing spermatozoa production in a continuum.

Vitamin A has been implicated for long in the spermatogenesis homeostasis and fertility. In the past two decades, new insights evidenced that retinoic acid drives the asynchronous initiation of spermatogonial differentiation, promotes the transition from preleptetone to leptotene spermatocyte and therefore the onset of male meiosis, and mediates postmeiotic transitions. Evidences showed that RA plays a broad and encompassing role in regulating and coordinating spermatogenesis. This chapter intends to provide an overview of the role of retinoic acid signalling in spermatogenesis.

Keywords: Spermatogenic cycle; Retinoids; Vitamin A; Spermatogenesis; Fertility

1. INTRODUCTION

Spermatogenesis is a complex, highly tuned and orderly process occurring in the seminiferous tubules in male testes. During spermatogenesis, diploid germ cells timely engage in the process of multiplication and differentiation that ends with the release of spermatozoa (a haploid cell) into the lumen of the seminiferous tubules (1). One round of spermatogenesis encompasses three stages: 1. the mitotic proliferation of spermatogoni (SPG), often named as spermatocytogenesis; 2. the reductional meiosis by spermatocytes (SPC); and the differentiation of spermatids (SPD), an unique maturational process during which an initial round cell transforms into a highly specialized flagellated cell with tightly condenced chromating and an acrosome – the spermatozoon (2, 3). The sequential progress of these three stages is tightly regulated in both time and space, under the orchestrated control of Sertoli cells, Leydig cells and the germ cells themselves (2), fostering a stage-related environment to the developing germ cells.

Postpubertal seminiferous tubules contain numerous generations of germ cells. Spermatogenesis in mammals relies upon a pool of undifferentiated spermatogonial stem cells (SSCs) residing in particular locations of the seminiferous tubules named niches that foster cell self-renewal (3). Niches are particular sites within the seminiferous tubules where the contribution from surrounding cells (e.g., Sertoli, myoid and Leydig cells) contribute to the creation of a particular environment that sustains SSCs differentiation into a pathway of self-renewal or proliferation (4, 5). In niches, SSCs have the unique capability to enrol in two different, exclusive pathways: to self-renewal (originating new spermatogonial stem cells) or to proliferate into more differentiated progeny (proliferating spermatogonia A). In the case of self-renewel, complete cytokinesis occurs with formation of two single cells, while in the proliferative differenciated SPG incomplete cytokinesis results in the formation of two cells connected by cytoplasmatic bridges (6). Novel generations of sperm cells arise from spermatogonia that are stimulated to differentiate into a proliferative profile without waiting for the preceding generations to complete their development and be released into the tubules (1), therefore ensuring that spermatogenesis is maintained throughout the male lifespan (7). The germ cells originating from an undifferenciated spermatogonium maintain a coordinated rate of differentiation, which is possible due to its syncytial arrangement during spermatogenesis (2).

SSCs recruitment into the differentiation pathway and meiosis occurs at time intervals, starting asynchronously through the seminiferous tubule (8), at space intervals, contributing to spermatozoal production *in a continuum* (1, 3). This particularity underlies the phenomena known as the spermatogenic wave, which has been demonstrated to exist in a large number of mammalian species (1). Recruitment of SSCs into the proliferative pathway supports two distinct events, as from that division, one of the cells returns to a quiescent, non-proliferative stage, allowing for the replenishment of the spermatogonial population, while the other further mitotically divide, committing to meiosis (1, 7).

The importance of Vitamin A and the retinoic acid as potential regulators of spermatogenesis and sperm maturation through the epididymis has been recognised for long (9, 10). Insufficient Vitamin A intake has been associated with infertility due to loss of most germ cells from the seminiferous tubules. Even though Vitamin A or retinoids administration may revert most symptoms of Vitamin A deficiency, the dynamics of spermatogenesis may be recovered, albeit in a synchronous pattern that disrupts the typical wave of spermatogenesis (10). Despite that treatment unblocks the spermatogonial differentiation and meiosis, it triggers a simultaneous differentiation of undifferentiated spermatogonia and therefore synchronous spermatogenesis (11), and also often increases the number of degenerated cells (12). Solid studies support the evidence that Retinoic Acid (RA) affects gene expression in the seminiferous epithelium, in a stage-specific pattern. Any stimuli impairing the retinoic acid signalling will negatively impact spermatogenesis and may lead to azoospermia (2). It has been shown that the trigger of SSCs into mitosis is rigidly timed and under the control of a peak in retinoic acid, whose testicular levels are strictly controlled (2, 6). This molecule has also been associated with the initiation of the meiotic process and spermiogenesis (3). Furthermore, retinoic acid is crucial for the first cycle of the seminiferous epithelium at puberty.

In this chapter, we intend to review the role played by the retinoic acid on spermatogenesis and discuss the current understanding about the retinoic acid signalling pathways in Sertoli cells and germ cells.

2. VITAMIN A AND RETINOIDS

Vitamin A is a term usually used as a generic descriptor for all molecules with the biological activity of retinol (13). Six isoforms of retinol are reported with biologic activity (14), but the all-*trans*-retinol (generally named retinol, ROL) is the most common and best studied in animal tissues. Retinoic acid (RA) and retinyl esters (RE) are derivatives of retinol and are often collectively referred as vitamin A (15). However, the term Vitamin A should refer only to all-*trans*-retinol, a compound that, when sufficiently present in the diet, satisfies all the vitamin A requirements for proper development and growth (16). Vitamin A-active compounds presenting the biological activity of retinol, comprise retinoids (retinol, retinaldehyde and retinoic acid) and provitamin A (carotenoids). Vitamin A is essential for vision and immune system as well as indispensable for the formation and maintenance of several animal tissues (17) (18). Vitamin A, after conversion to RA, also plays a role in reproduction and embryonic growth and development (19), affecting embryonic development and organogenesis, tissue homeostasis, cell proliferation, differentiation, and apoptosis (20, 21).

2.1. Retinoids metabolism

Most animals cannot synthesise Vitamin A. It is an essential micronutrient that must be acquired from the diet either as in the form of provitamin A carotenoids, occurring in vegetables and fruits, or as preformed retinoids (retinyl esters and retinol the most abundant forms) present in foods of animals (13). Animals cleave absorbed carotenoids to form retinal

(21). Alternatively, animals can obtain retinol by eating animal tissues that have already converted carotenoids to retinoids such as retinol and retinyl esters (14). Absorbed retinoids are secreted from enterocytes into the lymph either as chylomicrons (containing carotenoids, retinyl esters, small amounts of free retinol) and retinol and transport either to target tissues or hepatocytes for storage (15). Retinyl ester is the primary form store in the liver of mammals, birds, and fish) (21).

The retinyl esters are hydrolysed at the hepatocyte cell membrane and free retinol, which is then bound to a retinol binding protein (RBP). RBP are carrier proteins for retinol that, together with transthyretin, facilitate the transport the fat-soluble retinol in the bloodstream and the interstitial compartment of target-tissues (22). In vitamin A sufficient states, the retinol-RBP (holo-RBP) complex is secreted associated with transthyretin and transferred from hepatocytes to the stellate cells of the liver. Here it is esterified by LRAT to form mainly retinyl palmitate. The stellate cells contain 90% - 95% of hepatic vitamin A as cytoplasmic lipid droplets (23).

Retinol is delivered from the liver to target cells bound to RBP (holo-RBP) and transthyretin (24). Holo-RBP binds to cell surface RBP receptor STRA6 that mediates retinol (ROL) uptake from holo-RBP into cytoplasmatic CRBPI (25). CRBP is a cellular high-affinity retinol binding protein, which facilitates its transport into the nucleus or its metabolism in different cells (26). ROL is delivered to the intramembrane system (endoplasmatic reticulum) where ROL is esterified by lecithin retinol acyltransferase (LRAT) to RE or oxidised by alcohol dehydrogenases (ADH) to retinal (RAL). Finally, RAL are oxidised to RA by cytoplasmic retinaldehyde dehydrogenase (RALDH), or they are reduced back to retinol by retinaldehyde reductases (RalRD) in the internal membranes (27). In addition, RA could derive from β-carotene (CAR) uptaken by the cells, cleaved into two molecules of retinaldehyde by BCMO-1 (β, β carotene-15,15'-monooxygenase) and then oxidised into RA (27, 28).

In the cell, RA binds to CRABPI or CRABPII and is transferred by holoCRABPII to the nucleus to activate transcription (autocrine), transported to a nearby target cell (paracrine) or delivered to endoplasmatic CYP enzymes by holoCRABPI for further degradation (27). Tissue RA concentrations are regulated both by enzymes that generate RA (retinol and retinal dehydrogenases) and by enzymes that metabolise RA to fewer active metabolites, including cytochrome P450 enzymes from CYP26 family (CYP26A1, CYP26B, CYP26C1) (29, 30).

2.2. Tissue targeting and retinoic acid signaling

In the classic signalling mechanism (canonical pathway) retinoic acid effects are mediated through binding to specific nuclear receptors (retinoid receptors) followed by changes of transcription of several genes (31, 32). Retinoid receptors can be divided into two subgroups, RA receptors (RARs) and retinoid X receptors (RXRs) (33). The RAR has three isotypes (RARα, RARβ, and RARγ) (34), and each is capable of heterodimerizing with a retinoid X receptor isotype (RXRα, RXRβ, or RXRγ). The RAR–RXR heterodimer binds on the chromosome to sequence stretches called RA-response elements (RAREs) and functions as a transcriptional activator in the presence of its hormonal ligand and as a repressor in the absence

of RA (35). On the other hand, Retinoid X receptor (RXR) interacts with RAR, allow RXR to function as a master controller for signals from various hormonal pathways. In the nucleus, RAR and RXR form heterodimers bound to RAREs and associate with a co-repressor complex.

According to the canonical model of gene regulation by RARs (Figure 1), in the absence of RA, the DNA-bound RAR subtype represses target gene expression through the recruitment of corepressors such as nuclear receptor corepressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Several studies suggested that SMRT would be the RAR-favored corepressor (36, 37), which serve as molecular adaptors recruiting histone deacetylase (HDAT) (38). The co-repressor complex deacetylate lysine residues in the N-terminal tails of histones and induces chromatin condensation and repressed state over the target promoter (39, 40). Binding of RA induces a conformational change of the RAR/RXR heterodimer that results in the release of co-repressors and create a new interaction surface for co-activators which include p160 subfamily of steroid receptor coactivators (SRCs), namely SRC-1, SRC-2, and SRC-3 (29, 41). The p160 coactivators have intrinsic histone acetyltransferase (HAT) activity and acetylate several lysine residues in H3 and H4, initiating gene transcription (42).

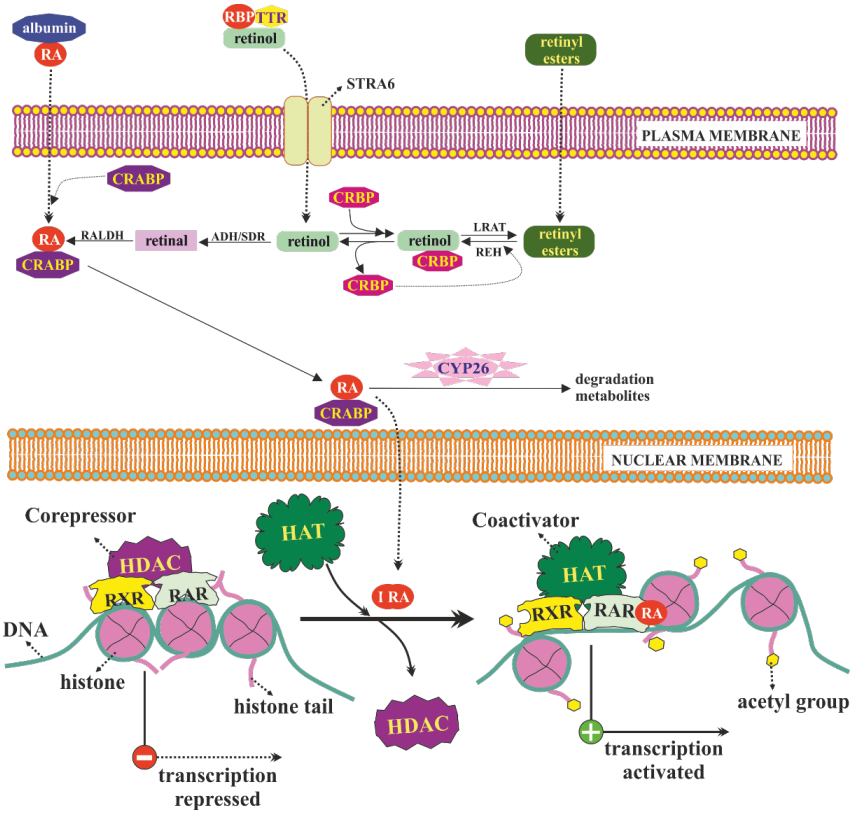

Figure 1. Gene regulation by retinoic acid nuclear receptors. The inactive heterodimer RAS-RXR is located in the nucleus and binds DNA. In the absence of retinoic acid, RAR-RXR is complexed with a corepressor HDAC, which prevents gene transcription. In the presence of retinoic acid, the heterodimer suffers conformational modification, decreasing its affinity for HDAC (that is released) and increasing the affinity for the coactivator HAT, activating the target gene expression. **ADH** – alcohol dehydrogenase; **CRABP** – cellular retinoic acid binding protein; **CRBP** – cellular

retinol binding protein; **CYP26** – cythochrome P450 family 26; **HAT** - histone acetyltransferase; **HDAC** - histone deacetylase; **LRAT** - lecithin retinol acyltransferase; **RA** – retinoic acid; **RALDH** - retinaldehyde dehydrogenase; **RAR** – retinoic acid receptor; **RBP** – retinol binding protein; **REH** – retinyl ester hydrolase; **RXR** – retinoid X receptor; **STRA6** – stimulated by retinoic acid gene 6; **TTR** – transthyretin.

Over than 500 genes have been put forth as being regulatory targets of RA. In some cases, direct regulation was demonstrated driven by ligand RAR/RXR heterodimers bound to RAREs (43). Retinoic acid induces the transcription of many genes encoding proteins that are involved in cell differentiation and a variety of biochemical processes (19, 44). First RAR target genes to be discovered includes RARβ, laminin B1, CRBP, and CRABP (29). Moreover, proteins involved in RA metabolism, such as CYP26, are also directly regulated by RA (43). The Hox gene, some of the most well-known target genes of RARs, are key regulators of pattern formation in vertebrates during development (45). RARs also regulate factors involved in metabolism (29).

In addition to its canonical effects (gene-mediated), retinoic acid also has some nongenomic (gene-independent) function. Non-canonical retinoid signalling is independent of gene transcription mediated by RAR/RXR heterodimers (27). Some of these non-genomic effects are the result of RA binding to retinoid receptors, but can often occur in the absence of retinoid receptors. RAR has also been implicated in mediating RA nongenomic actions (46). Evidence indicates that RA also utilizes other non-genomic pathways: phospholipase C-PKC, phosphatidylinositol-3-kinase (PI3K)-Akt (47), RAF-ERK-MAPK pathway or v-src sarcoma (SRC) tyrosine kinase activation (46, 48). PI3K activates PDK1, which in turn activates Akt. This kinase mediates mammalian target of rapamycin (mTor) activity, a serine/threonine protein kinase. mTor is the catalytic subunit of the mTORC-1 protein complex, integrating nutrient signals and mitogen promoting cell survival, growth and proliferation (49). RA activated-dependent PI3K/Akt pathway leads to phosphorylation of the tuberous sclerosis protein (TSC1), resulting in mTORC-1 activation. The downstream effectors of mTORC-1 are translational modulators within the protein synthesis pathway, ribosomal S6 kinase 1 (S6K1) and initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) which are stimulated by mTORC1-mediated phosphorylation, enhancers of protein synthesis, cell growth and proliferation (49). AKT also contributes to cell cycle progression and inhibition of apoptosis by increasing the expression of cyclins and anti-apoptotic Bcl-2 family members, mediating hyperphosphorylation of proapoptotic BAD, leading to the mitochondrial-dependent apoptosis (intrinsic apoptosis pathway) (50). Additionally, RA activates the ERK-MAPK signalling cascade and regulates mTORC-1 activity: ERK phosphorylates and activates kinase RSK1 that in turn phosphorylates and inhibits TSC1/2, followed by increased activity of mTORC-1 (29, 47).

3. EFFECTS OF RETINOIC ACID ON SPERMATOGENESIS

Several studies revealed that several crucial germ cell differentiation steps occur at a particular stage of spermatogenesis and all of them are driven by periodic increases in RA levels

(e.g., the stages VII to XII in the mouse model) (51). It has been shown that occurrence of all those steps is concentrated in the same spermatogenic steps, such as the differentiation of spermatogonia, meiotic initiation, acquisition of spermatid polarity and spermiation (51-53). From this point on, this review will concentrate on the effects of RA in those particular events of spermatogenesis. Most of the available information on the RA effects on spermatogenesis was gathered from rodent species. There is still a lot to explore about RA signalling pathways in other species.

3.1. Evidences of retinoid acid effects in spermatogenesis

The first study supporting Vitamin A requirements for normal spermatogenesis was published in 1925. This study showed that vitamin A deficiency induces an early arrest of spermatogenesis, which was later associated with degeneration of germ cells and defective testosterone secretion. These changes could be reversed by either vitamin A or retinoic acid administration (22). Lately, using transgenic animals, several experiments described the disruption of spermatogonial differentiation and further downstream effects of RA. Along with excess and depleted RA models, those studies allowed to clarify some RA signalling pathways and RA effects on spermatogenesis (11, 22).

In Vitamin A-deficient animals (VAD), it has been described the complete arrest of undifferentiated spermatogonia, that failed to enter the proliferative pathway that starts spermatogenesis. This arrest could be reversed by administration of retinol, but its administration only allowed for synchronisation of spermatogenesis and the loss of the wave dynamics typical of the spermatogenic process (54).

Studies in post-natal knockout mice showed that dietary depletion of vitamin A originates meiotic arrest due to the loss of the gene *Stra8* expression (55), which is indispensable to switch from mitosis to meiosis (30). The accumulation of undifferentiated spermatogonia also found in those animals (8) further aggravates the effects of the meiotic arrest on the loss of spermatogenesis.

Models using both genetic and metabolic impairment of RA signalling, particularly those resulting in changes (inhibition or stimulation) of RA receptors suggested that there are different pathways involved in retinoids signalling in spermatogenesis, which differ between testicular somatic cells and germ cells, and even different populations of germ cells (10). In the normal tissue, these diverse pathways and players contribute to the finely tuned spermatogenic process, but when the mainstream RA signalling is disrupted, the negative effects on sperm production and male fertility are also potentialized.

3.2. Expression of retinoids and retinoid receptors in the mammalian testis

In the testis, all the main cellular types (peritubular myoid cells, Sertoli cells, germ cells and Leydig cells) respond to retinoids (22). Peritubular cells capture retinol (ROL) coupling it with cellular retinol binding protein (CRBP) and secrete it bound to a new RBP towards the

Sertoli cells. Sertoli cells are able to take up and storage ROL, either from the blood circulation or the peritubular cells, and convert it into retinoic acid. RA production and storage within Sertoli cells varies with the stage of the seminiferous epithelium, associated with changes in FSH secretion (11). Inside Sertoli cells, RA is made available to germ cells and Sertoli cell functions, while the RBP-bound ROL can also be used by spermatocytes to synthesise RA (Figure 2). Besides the Sertoli cells, spermatids and testicular and epididymal spermatozoa can store retinoids (11). In the interstitium, Leydig cells can capture RBP-bound ROL secreted by the peritubular cells (22).

Cell compartmentalization of enzymes and proteins involved in RA synthesis, degradation, and storage, kept testicular RA levels tightly regulated – both temporally and spatially. In the seminiferous tubules, pulses or RA are produced in a stage-dependent manner, allowing to adapt RA levels to particular stages of sperm cells development. The peritubular myoid cell layer acts as a catabolic barrier, preventing RA from entering the seminiferous tubules. Also, late pachytene and diplotene spermatocytes can synthesise RA, providing the germ cells developing in the adluminal compartment with a complementary RA source (52, 56). It has also been demonstrated that germ cells express RA receptors according to their developmental stage (56).

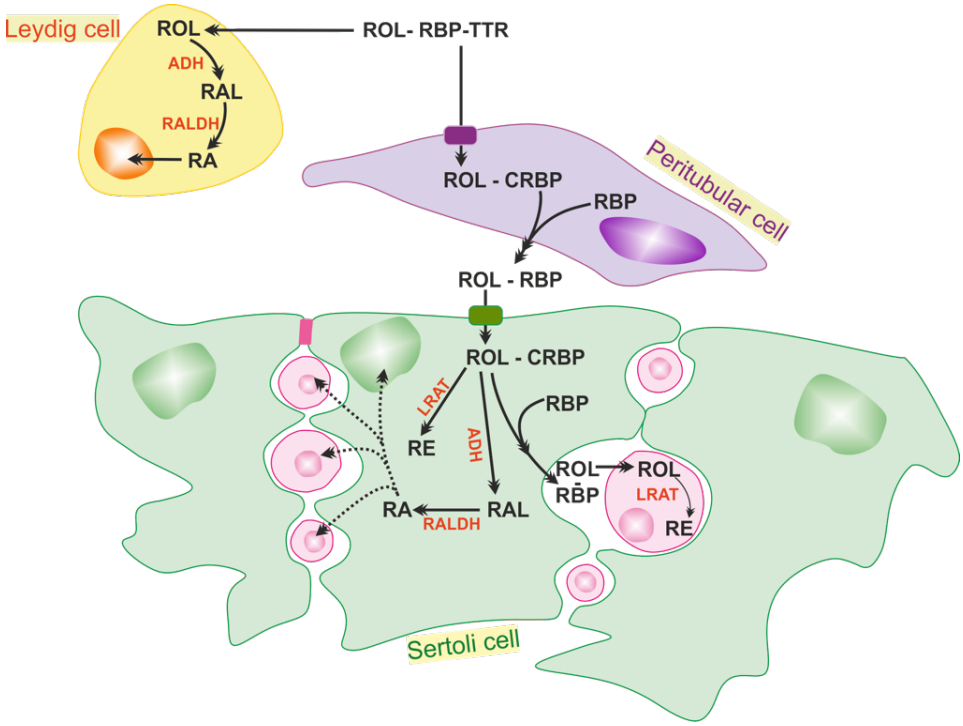

Figure 2. Regulation of testicular functions by retinoids. ADH – alcohol dehydrogenase; **CRBP** – cellular retinol binding protein; **LRAT** - lecithin retinol acyltransferase; **RA** – retinoic acid; **RALDH** - retinaldehyde dehydrogenase; **RAR** – retinoic acid receptor; **RBP** – retinol binding protein; **RE** – retinyl ester; **ROL** – retinol; **TTR** – transthyretin.

Table 1 summarises the information regarding the presence of retinoid receptors in mammal testis.

Table 1. Location of retinoid receptors and proteins in the testicular tissue

Testicular cells		Molecule
Sertoli Cells		CRBP-I CRABP-II [Fetal and early postnatal] RAR-α1, RAR-α2 RAR-β RXR-β
Peritubular myoid cells		CRBP-I RAR-α
Germ cells	Gonocytes	CRABP-I
	SPG	CRBP-I CRABP-I RAR-α RAR-γ
	SPC-I	CRBP-I RAR-α1 [SPC-pach] RAR-γ [SPC-pach]
	SPD-r	RAR-α1 RXR-α RXR-γ
Leydig cells		CRBP-I CRABP-I CRABP-II [Fetal and early postnatal] RAR-α RAR-γ RXR-β RXR-γ

SPG – Spermatogonia; **SPC-pach** – pachitene Spermatocytes; **SPD-r** – round Spermatids; **CRBP** – cellular retinol binding protein; **CRABP** – cellular retinoic acid binding protein; **RAR** – retinoic acid receptor; **RXR** – retinoid X receptor

3.3. Retinoic acid signalling pathways in Male Germ Cells and Spermatogenesis

a/ RA in fetal and prepubertal suspension of spermatogenesis

Albeit spermatogenesis often refers to the process of spermatozoa production that starts at puberty in the male, one could consider that it should also respect the embryonic period, when primordial germ cells migrate (PGCs) into the differentiating genital ridge (Figure 3). PGCs actively proliferate until they associate with somatic cells. Soon after the somatic sex differentiation in the gonad, the proliferating primordial germ cells gradually lose their mitotic ability and enter a quiescent phase, that is maintained until puberty (5, 57). The arrest of the proliferative ability and the inhibition to enter meiosis contrasts with the observed in the female gonad (58) and has been related to an inhibition the RA signalling cascades (59).

Evidence showed that in male gonads, after the indifferent gonad acquires a male-like morphological pattern, under the influence of SRY, the RA of mesonephric origin is sequestrated (metabolised) mainly by Sertoli cells. Consequently, the levels of RA are too low to trigger the production of STA8, which do not permit germ cells to initiate meiosis (59, 60). Several studies demonstrated that, in Sertoli cells, CYP26B1 degradates all-*trans*-RA into inactive metabolites, thereby locally regulating the RA levels (cf. (59). Similarly, in cultures of male foetal mouse gonads treated with CYP26B1 inhibitors, primordial germ cells enters meiosis, alike it occurs in the female gonad (61).

Gonocytes are the sole source of the functional SSCs pool in the postnatal testis (60). While maintenance of the SSC occurs from shortly after birth to the extent of adulthood, as a result of the self-renewal ability demonstrated by SSCs, spermatogonial differentiation from those undifferentiated cells start at the onset of puberty (Figure 3) (57).

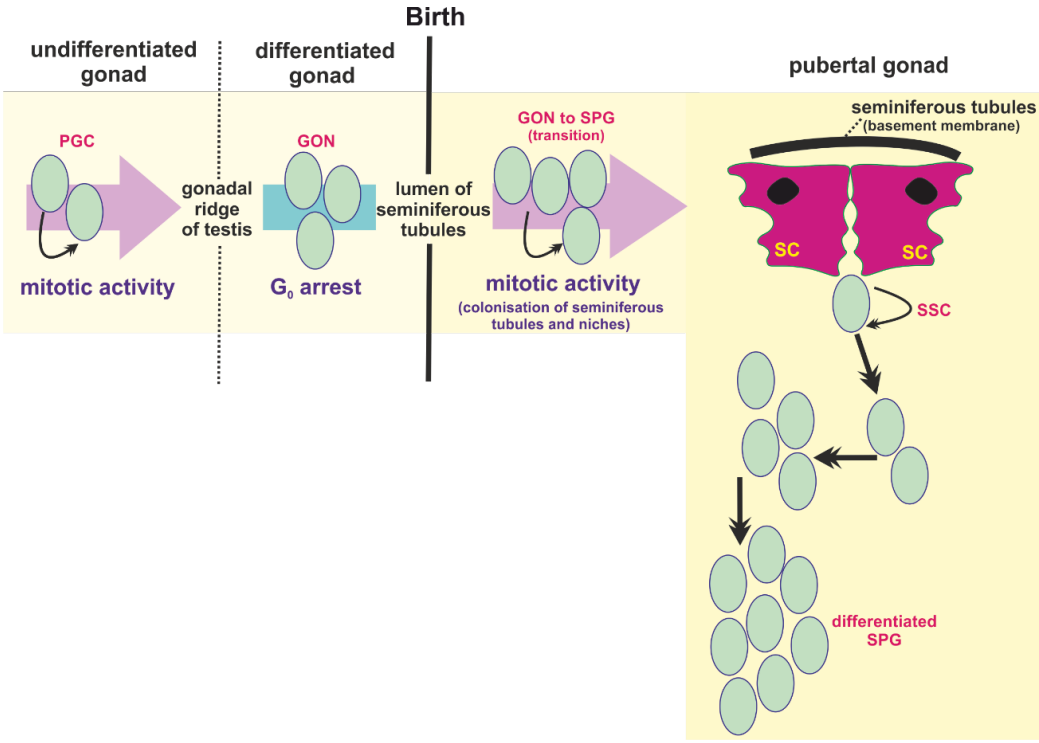

Figure 3. Overview of mitotic germ cell development in mammals. PGC – Primordial germ cells; **GON** – Gonocytes; **SPG** – Spermatogonia; **SSC** – Spermatogonial stem cells; **SE** – Sertoli cell.

b/ RA and the asynchronous SPG recruitment

Spermatogonial differentiation (or its exit from the stem cell pool), is one of the pivotal transitions in spermatogenesis (51). Once the pool of SSCs is established, in adult testes, differentiation of those cells can involve two types of process, as already said: the self-renewal pathway that produces progenitor cells, or the one originating differentiating spermatogonia

(62, 63). The second type of differentiation process depends on the retinoic acid signalling, which results in the expression of *c-Kit*, in the differenciated SPG (57, 62). When this type of differentiation occurs, the differentiated SPG leaves the niche and commit to spermatogenesis (57). The proliferation of differentiated spermatogonia is originated by binding of the Stem Cell Factor (SCF), produced by Sertoli cells, to the SPG existing *c-Kit*, and allow for the amplification of the number of cells acquiring the competency to enter meiosis (63). In mice, WNT-family polypeptides accelerate SSC proliferation (64).

Proliferation and differentiation of SSCs are regulated by intrinsic factors (the stem cell itself) and extrinsic factors (the niche environment). The intrinsic regulation of SSC fate remains poorly understood (63), but it may be possible that would command the ability of SSCs to respond to environmental cues to differentiation. Regarding the extrinsic factors, it is now accepted that, within the niche, endogenous, locally controlled RA gradients or pulses trigger the periodic differentiation of male germ cells (8, 53, 63), according to an asynchronous pattern of SSCs differentiation that foster a continuum of spermatozoa production through the male adult life.

The RA pulses results of its storage after synthesis from retinol by RDH or DHRS enzymes (53) in Sertoli and peritubular myoid cells. Activation of RDH or DHRS enzymes occurs in a stage- specific manner (Figure 4), contributing to the spatiotemporal control of RA biosynthesis. RA chaperoned by CRABP binds to its receptors. According to Mecklenburg and Hermann (63), it is not clear whether RARs or RXRs is the retinoid receptors involved in the cellular changes induced by RA in progenitor spermatogonia. In consequence, the nuclear response includes the translation of mRNAs encoding the KIT tyrosine kinase and STRA8, through a mechanism involving P13K/AKT/mTOR (65) and ERK signalling pathways (62).

While in the niche, SSCs are exposed to changes in the gradients of growth factors and cytokines, among other molecules, promoting the maintenance of the actual and potential germline stem cells in their undifferentiated state (57, 63), by leaving the niche, the differentiated SPG eludes their influences and engages in proliferation.

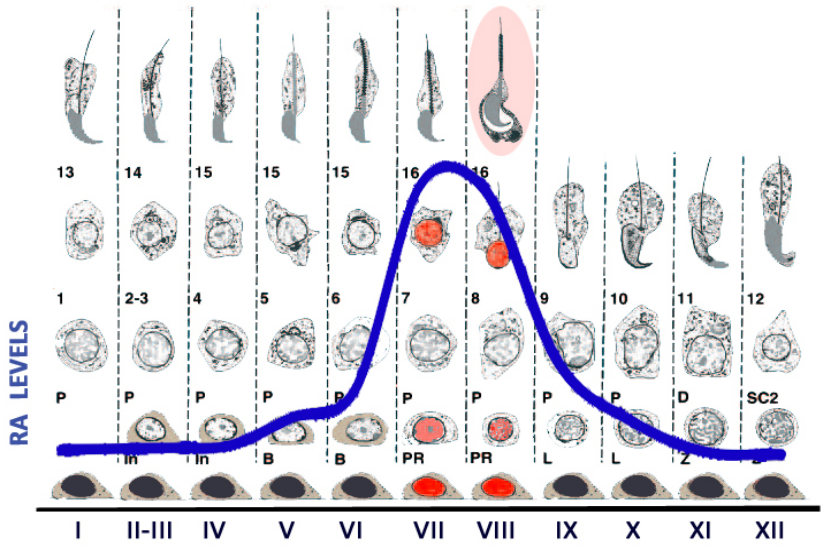

Figure 4. Schematic representation of the proposed RA relative levels (in blue) through the cycle of the seminiferous epithelium in mice, as per the pulse theory. Red colour signals the critical RA-dependent transitions reported in germ cells (spermatogonial differentiation from SSCs, meiotic competency, and acquisition of polarity in spermatids), which culminate in spermiation when mature elongated spermatids are freed to the lumen of the seminiferous tubules as single cells.

c/ RA in the initiation of meiosis

During spermatogenesis, the switch from mitosis to meiosis is dependent on the activation of the *Stra8* gene (8, 61, 66), an early RA responsive gene. STRT8 protein has been evidenced in pre-leptotene spermatocytes and is being considered crucial to the transition from type B spermatogonia/pre-leptotene to leptotene spermatocytes, and support progression of later stages of the meiotic prophase (67). Despite its dependence of RA, it seems that *Strat8* gene may also be under an epigenetic control that would contribute to its responsivity to RA stimulation (66). *Stra8* has been shown to be depressed by bHLH transcription factors, such as SOHLHs, *Nanos* (66), and DMRTs (68, 69), which is obtained by limiting all RA-induced transcription in general. However, Toyoda and collaborators (70) demonstrated that *Sohlh2* would interact with *Sohlh1* to control *Kit* synthesis (a marker of spermatogonia differentiation into preleptotene spermatocytes) through the transcriptional regulation of E-box, but did not found marked changes in *Stra8* transcription.

Star8 is crucial to the spermatocyte transition to the zygotene/pachytene stages. According to Ma et al. (66) *Stra8* regulates, directly or indirectly, the transcription of multiple genes, which relate to pre-meiotic DNA replication, double-strand break formation, chromosomal rearrangements and pairing, chromosomal synapsis, meiotic recombination processes and telomers binding.

Albeit the most accepted theory considers that *Stra8* is necessary and sufficient for initiation of meiosis in male germ cells, it has been recently suggested that other RA-induced players may be involved in the process (59). Koubova and colleagues (71) demonstrated that RA-dependent meiotic initiation uses both the *Stra8* and the *Rec8* independent pathways, both of them requiring the germ cells to express *Dazl. Rec8* is incorporated in the cohesin meiotic complex, essential during the chromosome segregation and chiasmata formation.

The increased *Stra8* gene expression has been hypothesized to be triggered, in the germ cells, by the canonic signalling pathway of RAR/RXR-RA and RARE (71). However, the RA-induced mechanisms of *Rec8* expression remained elusive.

d/ RA in spermatid development

RA signalling through RARα is essencial for spermatid polarization and orientation within the tubules. Failure in this pathway leads to a temporary arrest on SPD development (72) and an increase in apoptosis of elongating SPD (73).

It has been demonstrated that the actin filament bundles maintain spermatid polarity at the Sertoli cell–elongating/elongated spermatid interface at the apical ectoplasmic specialisations (74), which first appears when SPD starts polarises and elongation.

Rai14 is a retinoic acid-induced protein and an actin-binding protein that participates in the organisations of actin cytoskeleton filaments in the in the seminiferous epithelium. This protein has been implicated in the integrity of actin-based tight junctional complexes, in the basal ectoplasmic specialisations, that participate in the blood-testis barrier (75).

Disruption of Rai14 expression led to defects in the spermatid polarity, spermatid elongation and spermiation. Moreover, elongating and elongated SPD are also often found entrapped deeper in the seminiferous epithelium, at an inappropriate location for spermiation. It has been shown that disturbance of Rai14 would compromise the proper F-actin organisation, mediated by the binding to paladin, and consequently would impair the proper transport of SPD across the spermatogenic epithelium (76). Such defects in F-actin organisation was described either at the Sertoli-spermatid interface and also at the blood-testis barrier. Furthermore, RARα signaling participates in the control of cell adhesion. Hasegawa and Saga (77), in mice, demonstrated that RAR-mediated RA signaling is associated with a stage-dependent organization of the blood-testis barrier.

It has been shown that spermiation depends on RA signalling via the RARα/RXRβ heterodimer expressed in Sertoli cells (78). In consequence, it occurs the reorganisation of the actin filaments into highly branched networks forming the tubulobulbar complexes, transient endocytic structures through which the excess of spermatid cytoplasm is removed, contributing for the final disengagement of spermatids from Sertoli cells (79).

e/ RA and the blood-testis barrier

The spatial arrangement of germ cell within the tubules is crucial to spermatogenesis. This arrangement results of the particular junctional system created by both inter-Sertoli cell interactions and germ-Sertoli cell interactions. These different junctions create different functional compartments within the seminiferous tubules that serves the need for particular environmental conditions through the germ cell development while also creating an immune privileged site for the development of haploid germ cells. As to Peer et al. (3), RA signalling through RARα crosstalk with Sertoli cells to control the junctional processes that ensure the migration of germ cells from one compartment to another (52). This transmigration is accomplished by breaking the inter-Sertolian tight junctions ahead of them and reforming the junctions behind, creating a transient compartment (80). Alike in spermiation, an involvement of Rai14 has been proposed.

3.4. Influence of retinoic acid on sperm metabolism and oxidative stress

Free radicals and other reactive oxygen species (ROS) are continuously generated as byproducts of normal cellular metabolism. Cells accomodate ROS using scavenger molecules, including Vitamin A. Retinoids are potent scavengers of reactive oxygen species (81), playing a crucial function in the cellular redox balance in different tissues and situations.

Research on biological systems has shown that reactive oxygen species (ROS) are far more than the unavoidable by-products of oxygen metabolism and that they play essencial roles, when in low, controlled levels, as signaling molecules regulating biological processes (82).

Normal physiologic levels of ROS are essential for fertilization, through their involvement in processes such as capacitation, acrosome reaction, gamete chemotaxis, binding to the zona pellucida, and sperm-oocyte fusion (83-85). Increasing levels of P-Tyr proteins characterize sperm capacitation. Protein phosphorylation results from an increased activity of protein kinase A (PKA), activated by cAMP, which is driven by stimulation of adenylate cyclase (AC) in the presence of physiological ROS levels, specifically superoxide radical. High levels of P-Tyr proteins may also result from an inhibitory effect of ROS over the phosphotyrosine phosphatase (PTPase) (83, 86).

In spermatozoa, the normal ROS values are controlled by the cellular antioxidant system that consists of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase, and vitamin E (87, 88). SOD has been detected in the sperm of several species, including humans, rabbits, and mice (89, 90). Sustained SOD activity triggers increased levels of H_2O_2, which in turn has been implicated in ROS signaling, namely in the activity of protein phosphatases (PTPs), nonreceptor protein tyrosine kinases (PTKs), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and transcriptional factors (TFs) – all of them critical intracellular pathways of normal spermatozoa function (85, 91, 92).

An excessive production of ROS has been associated with sperm dysfunction (81, 85, 93). Lipid and protein peroxidation of sperm membranes, DNA damage and cell death (apoptosis) follow the increased in ROS concentrations, consequently affecting sperm concentration, motility, and morphology (94-99). In brief, they compromise male fertility, which agrees with the finding that superoxide dismutase activity is lower in the seminal plasma of infertile patients (100).

RA has been shown to mitigate tissue susceptibility to oxidative stress by acting on some oxidative stress enzymes, such as SOD, CAT and Glutathione reductase (101, 102). Retinoic acid impacts the oxidative stress-mediated apoptosis in several tissues (103-105) and embryo differentiation (106). Redox-mediated apoptosis could be reverted in those tissues by the administration of all-trans-retinoic acid (ATRA). Retinoic acid modulates antioxidant mechanisms also in spermatozoa (107). The action of RA in cell physiology is mediated by nuclear RA receptors and retinoid X receptors. Additionally, it has been described as a cytoplasmatic localization of receptors in bulls and dog sperm, probably playing an active role in the cross-talk with other signal transduction pathways (108).

Evidence showed that a broad spectrum of RA physiological effects migth result from both a receptor- and a non-receptor-dependent mechanism (109). Retinoic acid at physiological concentrations inhibits the decrease in SOD activity and significantly reduces hyperglycemia-induced oxidative stress, protects neurons from oxidative stress, and prevents a reduction in SOD levels in rats after heart overload with pressure (110, 111). Besides, RA mitigated changes in SOD protein levels decreasing the rate of SOD protein degradation in staurosporine-induced oxidative stress in neonatal rat hippocampus cells (102).

4. ABNORMAL RETINOIC ACID SIGNALLING AND HUMAN MALE INFERTILITY

Supported by the crucial role of retinoic acid in spermatogenesis, it has been hypothesized that infertile men have insufficient RA levels to sustain spermatogenesis due to impaired biosynthesis of retinoic acid (112). Reduced aldehyde dehydrogenase enzymes were found in testicular samples of men with idiopathic infertility associated with oligospermia or azoospermia (113). The decreased in ALDH was accompanied by a reduction of germ cells in the testicular tissue, particularly of the cells in post-meiotic stages of development (113). Later on, it has been shown that the intratesticular levels of 13-cis- retinoic acid were lower in men with oligoasthenozoospermia compared to healthy subjects, albeit the ATRA intratesticular levels remained at similar levels (112). The administration of 13-cis-retinoic acid (20mg/day BID for 20 weeks) improved the total sperm counts and the sperm morphology, while the motile sperm count per ejaculate was only slightly improved (114).

Besides, RAR or RXR loss-of-function in Sertoli cells have been related to spermiation failure and desquamation of immature germ cells, as well as with increased germ cell apoptosis, while the Sertoli cells losses their stage-dependent gene expression (115).

Albeit the studies supporting the assumption that certain types of infertility seem to correlate with lower concentrations of intratesticular retinoic acid (112, 114), a clear association between RA intratesticular levels and sperm impaired motility are yet to be established. It is, however, possible that such effects may be related to an alternative pathway, linking the RA signaling to oxidative stress, as previously mentioned. In recent years, it has been shown that ATRA administration ameliorates oxidative stress and apoptosis in diverse conditions mediated by toxicity or inflammation (116, 117), including in men varicocele related infertility (118). Besides the already mentioned interplay with oxidative stress enzymes and oxidative-induced apoptosis, it has been shown that RA affects cell membrane integrity and mitochondrial function (119), as well as DNA integrity (117). Dysregulation of RA signaling could, therefore, help to explain sperm impaired motility defects or excessive DNA damage, often associated with oxidative stress imbalance. This particular topic, however, has not yet been addressed when studying RA effects on sperm.

5. CONCLUDING REMARKS

Spermatogenesis is a highly complicated cellular differentiation processes, and a crucial determinant of male fertility. Spermatogenesis is regulated through a complex, multi-levelled process whereby diverse molecules play a critical role and enrol in crosstalk designed to ensure the continual production of spermatozoa. Retinoic acid is one of those molecules, and its importance has highlighted in the last decades. Available information on RA signalling pathways derives mostly from studies developed in rodent species. Despite that there are still much to learn, the progress made so far challenges researchers to find and develop a method that can support *in vitro* spermatogenesis. In particular, the culture of SSCs to be used for colonisation of seminiferous tubules would support the development of new techniques in the assisted reproduction area, mainly directed to restore fertility in young males submitted to

chemotherapy. In addition, it would enhance the knowledge in the mechanisms controlling spermatogenesis.

The relationship between RA imbalance and fertility remains elusive, but it may be related to the disruption of the oxidative stress balance, particularly in post-testicular or ejaculated spermatozoa. Further research on this topic is foreseen. Due to the particularities of the spermatozoon chromatin, it is possible the molecular pathways involved in RA signaling to be different from those reported to act in other cells.

We hope with this chapter to provide the reader with a concise reference on the retinoic acid signalling pathways in spermatogenesis and male (in)fertility.

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