Histol Histopathol (2022) 37: 1053-1064

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

REVIEW **Open Access**

How does retinoic acid (RA) signaling pathway regulate spermatogenesis?

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Summary. Male sterility is a worldwide health problem which has troubled many unfortunate families and attracted widespread attention in the field of reproduction. Retinoic acid (RA) is a metabolite of vitamin A. Previous studies have shown that insufficient intake of vitamin A can lead to male infertility. Similarly, RA-deficiency can lead to abnormal spermatogenesis in men. RA signaling is inseparable from hormone stimulation, such as FSH, testosterone and others. It can regulate spermatogenesis as well, including the proliferation and differentiation of spermatogonia, meiosis, spermiogenesis and spermiation. To promote or inhibit spermatogenesis, RA regulates Stra8, Kit, GDNF, BMP4 and other factors in various pathways. At the self-renewal stage, RA inhibits spermatogonia renewal by directly or indirectly inhibiting DMRT, GDNF and Cyclin. At the stage of differentiation and meiosis, RA controls SSC differentiation through Kit induction and Nanos2 inhibition, and controls spermatogonia meiotic entry through up- regulation of Stra8. At the stage of spermiogenesis, RARα, as a key regulator, regulates spermatogenesis by up regulating Stra8 while binding with RA. Although RA plays an important role in all stages of spermatogenesis, RA signaling is more important in the early stage of spermatogonia (spg) differentiation and spermatocyte (spc) meiosis. According to the principle of RA signaling that regulates spermatogenesis, we also speculate on the future clinical application of RA, such as potential induction of SSC *in vitro*, contraception and restoring spermatogenesis. This paper reviews the regulatory pathways of RA, and prospects the clinical applications of RA signaling in the future.

Key words: Spermatogenesis, Retinoic acid, Self-renewal, Proliferation, Differentiation, Stra8, GDNF, RAR

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DOI: 10.14670/HH-18-478

Introduction

Vitamin A, also named retinol (ROL), exists in the seminiferous tubules (S.T.) (Ahluwalia et al., 1975) , and can be hydrolyzed by alcohol dehydrogenases (ADHs)

Abbreviations. SCs, sertoli cells; GCs, germ cells; SSC, spermatogonial stem cell; PGC, primordial germ cell; GDNF, glial cell line-derived neurotrophic factor; FSH, follicle-stimulating hormone; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidyl inositol 3-kinase; ERK, extracellular signal-regulated kinases; CREM-1, cAMP response element modulation protein 1; PLZF, promyelocytic leukemia zinc-finger; Med1, mediator complex subunit 1; DMRT1, doublesex and Mab-3-related transcription factor 1; RA, retinoic acid; ATRA, all-trans RA; ROL, retinol; RAL, retinaldehyde; RALDH, RAL dehydrogenase; S.T., seminiferous tubules; ADHs, alcohol dehydrogenases; ALDH1A, aldehyde dehydrogenase enzymes of the 1A family; RDHs, retinol dehydrogenases; RDH10, retinol dehydrogenase 10; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; VAD, vitamin A deficiency; Cyp26, Cytochrome P450 family 26; Cyp26b1, Cytochrome P450 family 26 subfamily B member 1; BDADs, Bis-(dichloroacetyl)-diamines; RARs, retinoic acid receptors; RXRs, retinoid X receptors; RTR, retinoid receptor-related testis-associated receptor; FXR, farnesoid X receptor; ROR, retinoic acid receptor-related orphan receptors; MEK, mitogen-activated protein kinase kinase; Shp2, Src homologous domain tyrosine phosphatase 2; PKB, protein kinase B; SYCP3, synaptosome complex protein 3; TSPO, translocator protein 18kda; PCDH11Y, protocadherin 11 Y-linked; Gpat2, glycerol-3-phosphate acyltransferase 2; piRNAs, PIWI-interacting RNAs; CNNM1, cell cycle protein M1; miRNAs, microRNAs; TR2/4, testicular receptor 2 and 4; HYPO, hypospermatogenesis; SCOs, sertoli cell syndrome; MA, maturation arrest; SOD, superoxide dismutase; GST, glutathione transferase; ROS, reactive oxygen species; CDM, chemically defined media; BTB, blood-testis-barrier; Dmc1, disrupted meiotic cDNA 1; Rad51, recombination protein A; Smc3, Structural Maintenance Of Chromosomes 3; DAZL, deleted in azoospermia-like; TGCT, testicular germ cell tumors; KL, kit ligand; mTOR, mammalian target of rapamycin; FOXO1, forkhead box O1; Spz1, spermatogenic leucine zipper 1;Stra8, stimulated by retinoic acid 8; BMP4, bone morphogenetic protein 4; Sox9, SRY-box transcription factor 9; SRY, sex determining region Y; LH, luteinizing hormone; EE2, ethynyl estradiol 2; Stat3, signal transducer and activator of transcription 3; Sohlh, spermatogenesis and oogenesis specific basic helix-loop-helix; bHLH, basic Helix-Loop-Helix.

or retinol dehydrogenases (RDHs) to retinaldehyde (RAL) (Chaudhary and Nelson, 1984). Studies on cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP) show that both ROL and retinoic acid (RA) participate in maintaining testicular function, and the specific distinct binding sites for ROL and RA demonstrated in testicular nuclei and chromatin suggest their necessary functions during spermatogenesis in some cells of the testis (Ong et al., 1987).

The Sertoli cell is the main site of retinol uptake by the testis. In these cells, vitamin A can be either stored or oxidized to retinoic acid and, after binding to specific nuclear receptors, affect the expression of various genes (e.g. *stra8*). For example, when retinol is lacking, the activities of acrosin and plasminogen activator are greatly reduced which can return to normal after ROL supplementation (Zervos et al., 2005). So far, the main result of vitamin A deficiency (VAD) is spermatogenesis stagnation and testicular degeneration, and the oxidation of vitamin A by retinol dehydrogenase 10 (RDH10) to RAL is the key to biosynthesis (Tong et al., 2013).

RA is a normal metabolite of vitamin A. RA itself can be transformed into a metabolic form, which can promote the growth of vitamin A. Compared with vitamin A, RA metabolizes rapidly. RA has two types, trans-RA and cis-RA. All-trans RA (ATRA) and 9-cis-RA are the most important factors in spermatogenesis.

RA is transformed from RAL by aldehyde dehydrogenase enzymes of the 1A family (ALDH1A) proteins. This expression pattern is conserved in the developing male gonads of chicken (White Leghorn × Australop cross) and is dependent on Sox9 soon after SRY expression (Bowles et al., 2009). However, two kinds of RAL dehydrogenase (RALDH) in Sertoli cells (SCs) and germ cells (GCs) synthesize ATRA especially (Teletin et al., 2019).

LH and FSH (Nourashrafeddin, 2015), EE2 and xenoestrogens (Wang et al., 2019a) promote the synthesis of RA. Testosterone provides the environment for RA production and promotes RA synthesis (Wang et al., 2019b).

RA can be decomposed into 18-OH-RA or 4-OH-RA and 4-oxo-RA by Cytochrome P450 family 26 (Cyp26) family (Whitmore and Ye, 2015). CYP26 activity in Sertoli cells and germ cells is essential for the normal

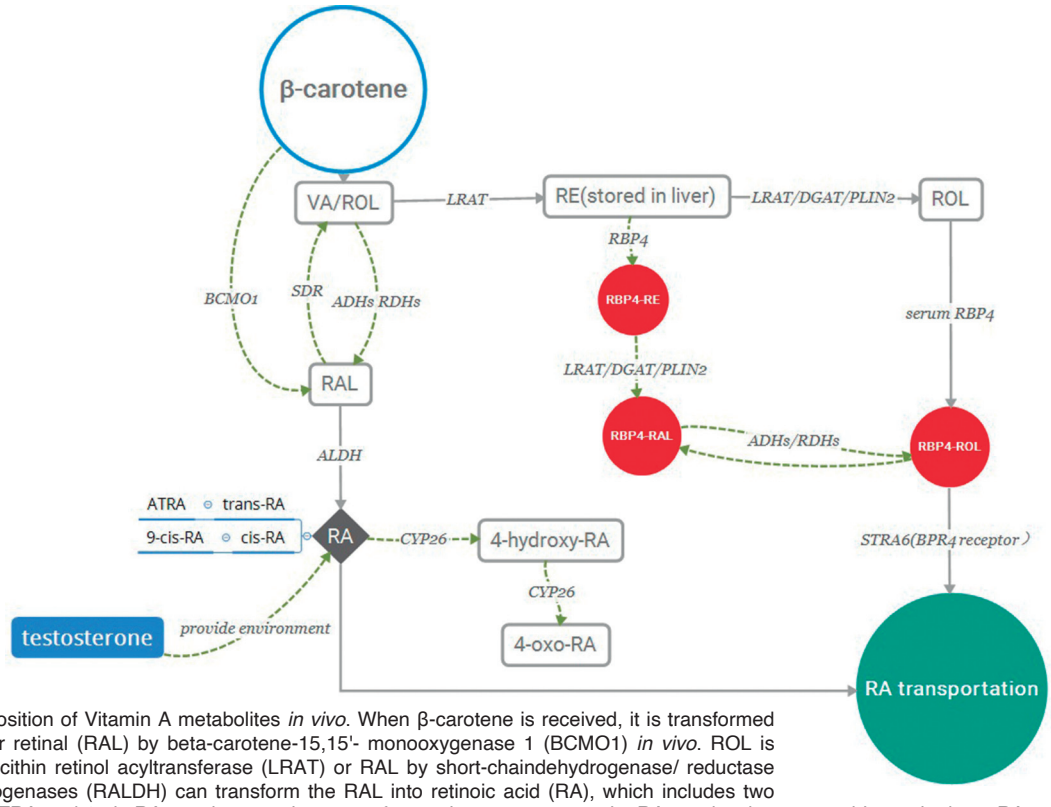

Fig. 1. Synthesis and decomposition of Vitamin A metabolites *in vivo*. When β-carotene is received, it is transformed into retinol (ROL/Vitamin A) or retinal (RAL) by beta-carotene-15,15'- monooxygenase 1 (BCMO1) *in vivo*. ROL is changed into RE in liver by lecithin retinol acyltransferase (LRAT) or RAL by short-chaindehydrogenase/ reductase (SDR). Retinaldehyde dehydrogenases (RALDH) can transform the RAL into retinoic acid (RA), which includes two types, trans-RA and cis-RA. ATRA and 9-cis-RA are the most important factors in spermatogenesis. RA can be decomposed into 4-hydroxy-RA and then 4-oxo-RA by cytochrome P450 family 26 (CYP26). RE can transform into RBP4-ROL in two ways. The first way is to become ROL first by LRAT/DGAT/PLIN2, and then be combined with serum retinol binding protein 4 (RBP4). The second way is to combine RBP4 first and then be transformed by LRAT/DGAT/PLIN2 into RBP4-RAL and can be mutually transformed with RBP4-ROL. With the help of STRA6, the membrane receptor of RBP4, RBP4-ROL can be transported across the membrane and change into RA.

process of spermatogenesis. The loss of CYP26 activity will lead to a decline of male fertility (Hogarth et al., 2015). Interestingly, ATRA can down-regulate the synthesis of Cytochrome P450 family 26 subfamily B member 1 (Cyp26b1) (Wang et al., 2019b).

So far, at least seven direct metabolites of RA have been found (CRABP-RA, retinoyl-coa, ALB-RA, retinoyl-glucuronide, 4-hydroxy-RA, 18-hydroxy-RA, rac-5,6-epoxy-RA), and 4-hydroxy-RA can continue to be metabolized inyo 4-oxo-RA and rac-4-hydroxy-4-o-beta-d-glucuronide-RA. (Whitmore and Ye, 2015). 4-oxo-RA was previously shown to be a more potent inducer of spermatogonial proliferation than ATRA. However, ATRA by itself is an active retinoid in spermatogenesis and does not need to be metabolized to 4-oxo-RA (Gaemers et al., 1997).

Bis-(dichloroacetyl)-diamines (BDADs) are compounds that inhibit spermatogenesis by blocking the metabolism of vitamin A. A specific BDAD, WIN 18446 can inactivate it by binding to the catalytic domain of ALDH1a2 (in rats, humans and zebrafish) with equivalent potency, thus regulating the production of endogenous RA in testis (Pradhan and Olsson, 2015). WIN 18446 also inhibited the transformation of ROL to RA in the ovary and testis of embryos cultured for 48 h (Hogarth et al., 2011).

RA signaling in mitosis

In spermatogonia, cyclin D(1) and D(3) are involved in the regulation of the cell cycle, promoting mitosis, while Cyclin D(2) was strongly induced in these cells after the induction of differentiation of most of these cells into A(1) spermatogonia by administration of retinoic acid (Ravnik et al., 1995; Beumer et al., 2000).

There are many regulatory miRNAs expressed in germ cells and Sertoli cells, among which miR-146, miR-222/223, miR-17-92, miR-16b-25, miR-471, miR-100 and miR-202 are regulated by retinoic acid (Walker, 2022). miR-100 is predominantly expressed in undifferentiated murine spermatogonia, including spermatogonial stem cells (SSCs). RA inhibited the expression of miR-100, which promoted SSCs proliferation by indirectly regulating Stat3 (Huang et al., 2017). How RA regulates miR-146, miR-222/223, miR-17-92, miR-16b-25 and miR-202 will be discussed in detail in the third part *RA signaling in meiosis*.

There are two types, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The former can bind RA or 9-cis RA, while the latter is only activated by 9-cis RA (Chung and Wolgemuth, 2004). There are three subtypes of RAR (RARα, RARβ, RARγ) and RXR (RXRα, RXRβ, RXRγ) in each receptor group. RARs and RXRs bind to RA response element, rare in heterodimers, which is located in the regulatory region (such as promoter) of RA target gene (Chung and Wolgemuth, 2004). If RA binds to RAR, RAR/RXR releases inhibitory protein complexes, activates target genes such as Stra8 transcription (regulated by RARα), or promotes the opening of the PI3K/PDPKI/AKT pathway and promotes the expression of mTORC1 and enhances the translational efficiency of repressed mRNAs required for spermatogonial differentiation (e.g. Kit, Sohlh1, and Sohlh2) through activation of kinase signaling (Busada and Geyer, 2016).

In marbled newt (*Triturus marmoratus marmoratus*), RXRs and FXR participate in the regulation of spermatogenesis by regulating the proliferation of primordial germ cells and spermatogonia (Alfaro et al., 2002).

RA signaling in meiosis

RA/RA wave

RA signaling regulates the expression of its direct target genes (including replication dependent core histone genes) to regulate the differentiation of spermatogonia. It is the key factor controlling the sex-specific timing of meiotic initiation in mammals, birds and tetrapods (Peng et al., 2020). The damage of RA signaling blocks the differentiation of spermatogonia, which indicates that the effect of RA signaling on the differentiation of spermatogonia *in vivo* can be directly targeted at the spermatogonia (Chen et al., 2016). *In silico* gene knockout and loss of hormone-sensitive lipase (Lipe) in ROL pathway can disturb RA wave and lead to a spermatogenesis defect (Whitmore and Ye, 2015).

Stra8 and kit

RA induces the transition of undifferentiated to differentiated spermatogonia, which is accompanied by the expression of genes such as stimulated by RA gene 8 (*Stra8*) and *c-kit* (Anderson et al., 2008 ; Zhou, et al., 2008a,b; Koubova et al., 2014; Busada and Geyer, 2016; Griswold, 2016). *Stra8* is a key gene in the initiation of meiosis in mammals and birds (Wang et al., 2017). Only when germ cells are activated by RA and express *Stra8*, can they enter meiosis (Baltus et al., 2006; Bowles et al., 2006; Smith et al., 2008; Bowles et al., 2010). RA dramatically stimulates *Stra8* expression in undifferentiated spermatogonia but has a lesser impact in differentiating spermatogonia (Zhou, et al., 2008a,b). Mitogen-activated protein kinase kinase (MEK) 1/2 activation was required during F9 cell differentiation towards somatic lineage, whereas its inhibition potentiated RA-induced *Stra8* expression, suggesting that MEK1/2 acts as a lineage specification switch in F9 cells (Manku et al., 2015). In adult mice, the expression of *Stra8* was limited to the germ cells before meiosis (Oulad-Abdelghani et al., 1996). Therefore, STRA8 protein may play a role in the early stage of spermatogenesis. The expression of *Stra8* can promote the expression of Src homologous domain tyrosine phosphatase 2 (Shp2). *Shp2* gene can promote the phosphorylation of extracellular regulated protein kinase

(ERK) and protein kinase B (PKB/Akt), and the expression of synaptosome complex protein 3 (SYCP3) and Dmc1. These meiotic genes such as Dmc1, Rad51 and Smc3 regulate the transformation of spermatogonia to spermatocytes, mediate the meiotic process and promote spermatogenesis (Li et al., 2020). miRNA-31 regulates the proliferation, DNA synthesis, and apoptosis of human SSCs by the PAK1-JAZF1-cyclin A2 pathway (Fu et al., 2019), overexpression of miR-31 can directly target *Stra8* and significantly inhibit spermatogenesis and destroy cSSCs in Rugao yellow chicken (Wang et al., 2017). The expression of SYCP3 and DAZL decreased with the decrease of STRA8 (Childs et al., 2011). DAZL encodes a germ cell specific RNA binding protein that induces *Stra8* and the onset of meiosis (Kasimanickam and Kasimanickam, 2014). Translocator protein 18kda (TSPO) is highly expressed in Leydig cells, and it has an inhibitory effect on differentiation. RA inhibited the expression of TSPO and promoted the expression of *Stra8* in TGCT cell lines (Manku and Culty, 2016).

c-kit plays an important role in the proliferation, migration, survival and maturation of spermatogenic cells. *c-kit* regularly expresses from PGCs to SSCs. After RA stimulation, the expression profiles of *c-kit* in testis and spermatogonial stem cell lines increased first and

Table 1. Upstream regulators and downstream factors of RA.

upstream regulators		materials	references
up regulate	LH	mice	Nourashrafeddin, 2015; Nourashrafeddin and Rashidi, 2018
	FSH	mice, *Danio rerio*	Huang et al., 1983; Chen and Liu, 2015; Nourashrafeddin, 2015; Nourashrafeddin and Rashidi, 2018; Crespo et al., 2019
	testosterone	*in vitro* (mice)	Sanjo et al., 2018
	T3	*in vitro* (mice)	Sanjo et al., 2018
	BMP4	mice	Yang et al., 2016
	FGF	mice	Pui and Saga, 2017
synthesize	RALDH/ ALDH1A1.2.3	rats. dogs. mice. Chicken (White Leghorn × Australop cross)	Duester, 2001; Bowles et al., 2009; Kasimanickam, 2016
degrade	CYP26A1.B1.C1	dogs	Bowles et al., 2009; Kasimanickam, 2016
down regulate	WIN18446	mice	Hogarth et al., 2013; Chen et al., 2018
	DMRT1	mice	Matson et al., 2010
downownstream factors		materials	references
up regulate	CYP26B1	dogs, Ochotona curzoniae	Kasimanickam and Kasimanickam, 2013; Yu et al., 2019
	BMP4	mice	Yang et al., 2016
	PDGFR	rats	Manku et al., 2015
	CNNM1	mice	Chandran et al., 2016
	stra8	human. rats. mice, marsupials(tammar)	Miyamoto et al., 2008; Manku et al., 2015; Hickford et al., 2017
	Prm1	mice	Silva et al., 2009
	Sycp1	mice	Silva et al., 2009
	Dazl	mice	Silva et al., 2009
	Act	mice	Silva et al., 2009
	Dmrt1	dogs	Kasimanickam and Kasimanickam, 2014
	Tnp1	*in vitro* (Chinese experimental miniature pigs)	Yu et al., 2019
	Piwil1	Langshan chicken	Xu et al., 2016
	Piwil2	mice	Silva et al., 2009
	PI3K/AKT/mTOR	mice	Busada et al., 2015; Serra et al., 2017
	PCDH11Y	*in vitro*	Anilkumar et al., 2017
	RHOX10	*in vitro*	Song et al., 2012
	RHOX13	mice	Busada and Geyer, 2016
	RAR/RXR	mice	Gaemers et al., 1997
down regulate	SOD/GST	*in vitro*	Malivindi et al., 2018
	GDNF	*in vitro*	Pellegrini et al., 2008
	Nanos	mice, *in vitro*	Lolicato et al., 2008; Yu et al., 2019
	Dppa3	mice	Silva et al., 2009
	Sycp3	mice	Silva et al., 2009
	Msy2	mice	Silva et al., 2009
	Tex14	mice	Silva et al., 2009
	Mir-17-92（Mirc1）	*in vitro*	Tong et al., 2012
	Mir-106b-25（Mirc3）	*in vitro*	Tong et al., 2012
	miR-202	mice	Chen et al., 2017
	miR-34c	goats, *in vitro*(mGSCs)	Li et al., 2013
	miR-146	mice	Huszar and Payne, 2013

then decreased, which was similar to the development of male germ cells *in vivo* (Zhang et al., 2013).

Spermatogonia can enter meiosis only after undergoing a KIT dependent division. miR-221/222 maintains undifferentiated mammalian spermatogonia by inhibiting KIT expression (Yang et al., 2013). ATRA increased KIT expression in spermatogonia and KL expression in Sertoli cells. ATRA and KL can increase the expression of Stra8 and Dmc1. ATRA and KL induce meiosis through the activation of PI3K and MAPK pathways through kit self-phosphorylation (Pellegrini et al., 2008). miRNA-26b can induce the transformation from Kit- to Kit+, and inhibits the proliferation and differentiation of spermatogonia. Plzf, the key transcription factor of undifferentiated spermatogonia, is the direct target of miRNA-26b. RA increases miRNA-26b and induces spermatogonia differentiation. miR–544 down-regulates the expression of PLZF, which directly affects the self-renewal and differentiation of male reproductive stem cells.

RA stimulates the PI3K / Akt / mTOR kinase signaling pathways and promotes spermatogonia differentiation (Serra et al., 2017). The PI3K/Akt/mTOR signaling pathway is crucial to many aspects of cell growth and survival, in physiological as well as in pathological conditions. PI3Ks constitute a lipid kinase family. Akt kinases belong to the AGC kinase family, related to AMP/GMP kinases and protein kinase C. mTOR is a key protein, evolutionarily conserved from yeast to man and is essential for life. Rapamycin inhibited mTORC1. Upon ligand binding, phosphorylated tyrosine residing in activated RTKs will bind to p85, then release the catalytic subunit p110. Activated p110 phosphorylated the PIP2 into the second messenger PIP3, and this reaction can be reversed by the PI3K antagonist PTEN. PIP3 will recruit the downstream Akt to inner membranes and phosphorylates Akt on its serine/threonine kinase sites (Thr308 and Ser473). Activated Akt is involved in the downstream mTORC1 mediated response to biogenesis of protein and ribosome (Porta et al., 2014; Follo et al., 2015). Rapamycin also blocked RA-induced translation activation of mRNAs encoding Kit, Sohlh1, and Sohlh2, but did not affect the expression of *Stra8* (Busada et al., 2015). In addition, the PI3K/Akt pathway also promotes spermatogonia differentiation. MEK/ERK is another pathway that can promote FOXO1 expression. Both pathways are downstream of GDNF and RA signaling and can be stimulated by FGF (Pui and Saga, 2017).

Doublesex-related transcription factor 1 (DMRT1)

DMRT1 is the control point for spermatogonia to enter meiosis. It is highly expressed in undifferentiated spermatogonia, less expressed in c-kit positive differentiated spermatogonia, and not expressed in pre-spermatocytes or other meiotic or post-meiotic cells. DMRT1 blocks meiosis by inhibiting RA directly and indirectly by inhibiting *Stra8* (Don et al., 2011). Meanwhile, DMRT1 activates the transcription of Sohlh1, which prevents meiosis and promotes the development of spermatogonia (Matson et al., 2010). DMRT plays an important role in both meiosis and mitosis, but how DMRT1 regulates the mitosis/ meiosis transition is not clear. Even if there are data that suggest miRNAs regulate the meiosis of SSCs through *Stra8*, there is no evidence to support this assumption (Wang et al., 2017).

Protocadherin 11 Y-linked (PCDH11Y)

Protocadherin 11 Y-linked, a member of the cadherin superfamily, is up-regulated by RA signaling transduction and plays an important role in the initiation of spermatogonia differentiation and meiosis. PCDH11Y mediates Wnt signaling transduction, and the down regulation of Wnt signaling leads to a down-regulation of Wnt target C-Myc and C-Jun (Anilkumar et al., 2017), which further leads to the difficulty of spermatogenesis.

Glycerol-3-phosphate acyltransferase 2 (Gpat2)

The expression of *Gpat2* is related to spermatogenesis, and RA can increase the expression of *Gpat2* (Garcia-Fabiani et al., 2015). GPAT2 is essential for the synthesis of piRNAs. PIWI-interacting RNAs (piRNAs) are miRNAs that protect the genome of germ cells from the influence of reversible transposable factors (Aravin et al., 2007) by binding to PIWI proteins (Juliano et al., 2011). PIWIL1 is one of the human PIWI proteins (Meseure et al., 2020), which play multiple roles in germline stem cell maintenance and self-renewal (Pammer et al., 2020). The PGCs from chicken (Langshan chicken) treated with RA showed that *Piwil1* ensured stable meiosis of germ cells during spermatogenesis (Xu et al., 2016).

NanoS

NanoS has RNA binding activity and an evolutionary conservation function in germ cell development. In mice, three nanohomologues were identified: NANOS1, NANOS2 and NANOS3. NANOS3 was found only in testis after birth. NANOS3 targeted destruction resulted in the complete loss of germ cells in both sexes. ATRA significantly down-regulated its expression. NANOS3 maintains undifferentiated spermatogonia by regulating its cell cycle (Lolicato et al., 2008). RA can also promote the differentiation of spermatogonia by inhibiting NANOS2, but GDNF and FGF9 promote the expression of NANOS2. RA inhibits the occurrence of GDNF, but FGF promotes RA and GDNF. FSH directly promotes GNDF and indirectly increases the effect of RA by promoting BMP4. miR-34c can inhibit the expression of NANOS2 and promote the differentiation of mouse spermatogonial stem cells (Zhang et al., 2012).

Exogenous BMP4 itself did not induce the expression of Stra8 and c-Kit, the two marker genes of spermatogonia differentiation, but BMP4 and RA had a significant synergistic effect, inducing each other's expression, thus promoting the differentiation of spermatogonia (Yang et al., 2016) . BMP4 was down-regulated in freshly isolated germ cells and VAD testes by retinol, but not retinoic acid (Baleato et al., 2005).

Spz1

bHLH-Zip gene *Spz1* is specifically expressed in testis and epididymis (Sha et al., 2003). Testosterone and RA down-regulate the expression of Spz1. This nuclear transcription factor, like other bHLH-zip molecules, binds to specific DNA sequences to regulate cell proliferation or differentiation, thus playing an important role in spermatogenesis (Hsu et al., 2001).

Cell cycle proteins

In spermatogonia, cyclin D(1) and D(3) are involved in the regulation of the cell cycle, and cyclin D(2) may play a role in the differentiation of spermatogonia (Beumer et al., 2000). Cell cycle protein M1 (CNNM1) is expressed in mouse testis from neonatal to adult, which is limited to spermatogonia in middle and early stage of testis. RA can down-regulate the expression of CNNM1 in GC1-spg cells, while the down-regulation of CNNM1 can trigger the differentiation of spermatogonia (Chandran et al., 2016).

miRNA

MicroRNAs (miRNAs) expressed in Sertoli cells and germ cells have been shown to regulate their proliferation and differentiation.

Knockout of Dicer of the key enzyme specifically expressed in mouse germ cells, resulted in the loss of miRNA expression, which led to development defects of germ cells and a decline of individual fertility (Yu et al., 2005). This indicates that miRNA plays a very important role in the proliferation and differentiation of germ cells.

Mirlet7 family miRNA was expressed in mouse spermatogonia and spermatocytes. RA induced Mirlet7-miRNA expression by inhibiting Lin28. The increased expression of the let-7 miRNAs results from the binding

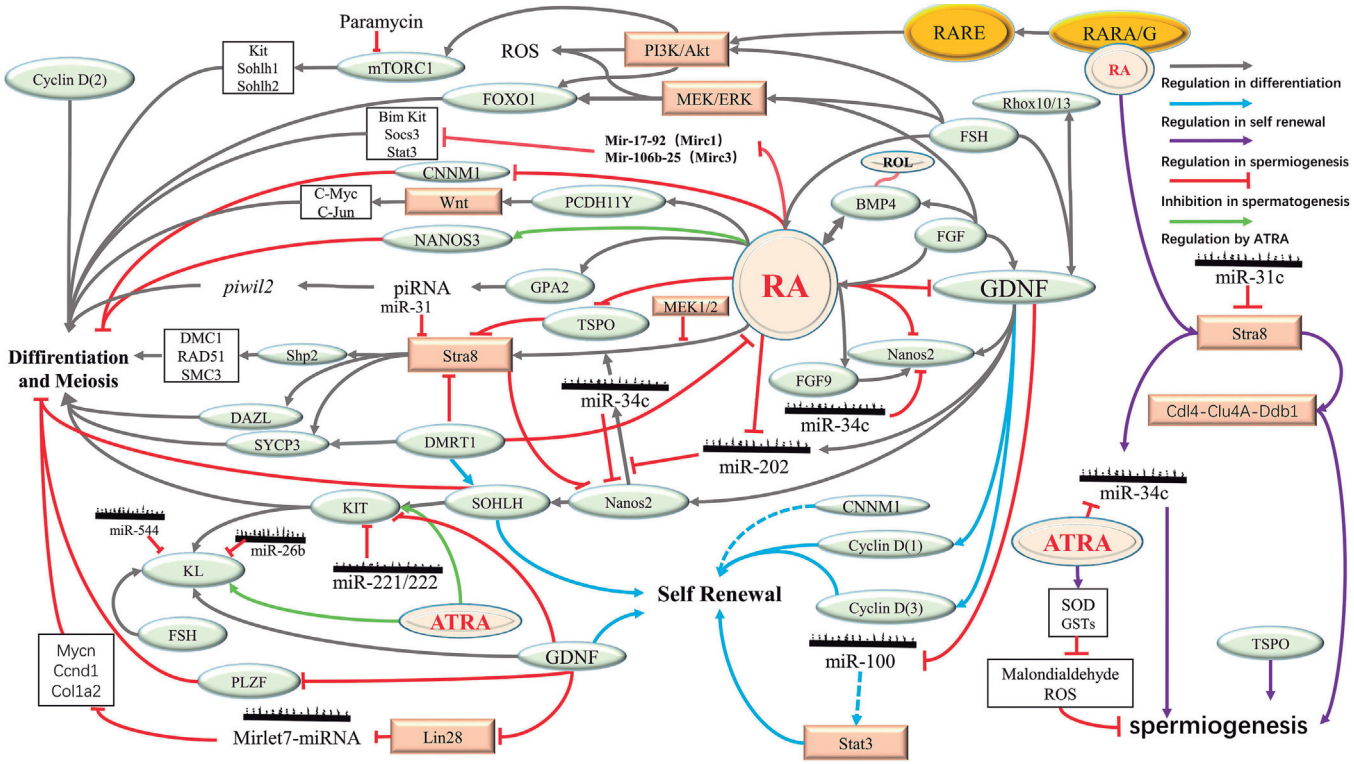

Fig. 2. RA signaling in spermatogenesis by pathways. RA signaling plays an important role in spermatogenesis. It regulates some factors like Stra8, Kit, GDNF, BMP4, etc. and then promotes or inhibits spermatogenesis through various pathways. RA regulates spermatogenesis in three stages: spermatogonia self-renewal (proliferation), differentiation and meiosis, and spermiogenesis. At the self-renewal stage, RA inhibits spermatogonia renewal by directly or indirectly inhibiting DMRT, GDNF and Cyclin. At the stage of differentiation and meiosis, RA controls SSC differentiation through Kit induction and Nanos2 inhibition, and controls spermatogonia meiotic entry through up regulation of Stra8. At the stage of spermiogenesis, RARα, as a key regulator, regulates spermatogenesis by up regulating Stra8 while binding with RA. In conclusion, RA is more important in the early stage of spermatogonia (spg) differentiation and spermatocyte (spc) meiosis instead of other periods like self-renewal or spermiogenesis.

of liganded retinoic acid receptors (RARs) to the promoter regions of the miRNA genes and the RARs acting as transcription factors to increase miRNA production (Zhong et al., 2010). The expressions of Mycn, Ccnd1, and Col1a2, which are targets of Mirlet7, were downregulated during spermatogonial differentiation both *in vitro* and *in vivo*. These results suggest that Mirlet7-miRNAs play a role in the differentiation of spermatogonia induced by RA (Tong et al., 2011).

miR-202 is highly expressed in mouse SSCs and is regulated by GDNF and RA, which are the key factors for self-renewal and differentiation of SSCs. It was found that miR-202 knockout SSCs initiate early differentiation, decrease stem cell activity and increase mitosis and apoptosis (Chen et al., 2017), which clearly shows the important role of miR-202's in promoting meiosis.

MiR-17-92 (Mirc1) and mir-106b-25 (Mirc3) miRNAs may play a synergistic role in the development of spermatogonia (Tong et al., 2012). In studies where RA induced spermatogonia differentiation, members of miR-17-92 (Mirc1) and its paralog mir-106b-25 (Mirc3) clusters were significantly down regulated. RA inhibits miRNA clusters miR-17-92 (Mirc1) and mir-106b-25 (Mirc3) (THY1+SSCs), which may increase the expression of Bim (Bcl2l11), Kit, Socs3 and Stat3. Male germ cell specific miR-17-92 (Mirc1) knockout mice have smaller testes, fewer epididymal sperm, and mild spermatogenesis defects. The deletion of miR-17-92 (Mirc1) significantly increased the expression of mir-106b-25 (Mirc3) miRNA in male germ cells. Some members of the miR-17-92 cluster may be critical players in spermatogenesis, including miR-17, miR-18a, and miR-20a. The *in situ* hybridization analysis of adult testes revealed that miR-17 is highly expressed in early stages of germ cells and is greatly decreased as germ cells mature, and miR-20a is mainly detected in the spermatogonia and in preleptotene spermatocytes (Olive et al., 2013).

Overexpression of miR-146 was sufficient to block retinoic acid-mediated differentiation of the spermatogonia by inhibiting Med1 expression (Huszar and Payne, 2013).

Retinoic acid also increases the expression of miR-10a. Overexpressing miRNA-10a in germ cells can make mice infertile and increase the differentiation of undifferentiated spermatogonia and decrease the number of spermatogonia entering meiosis (Niu et al., 2011).

Potential factors

It was mentioned in a study that testicular receptor 2 and 4 (TR2 / 4) are a subclass of orphan nuclear receptors, and play an important role in spermatogenesis. Both ROL and RA can promote TR4. These findings suggest that TR4 is a ligand regulated nuclear receptor and that RA may play a more extensive regulatory role by activating orphan receptors such as TR4 (Zhou et al., 2011). However, the study of this pathway is not clear. Similarly, the role of ROR receptor in the regulation of spermatogenesis has been mentioned in recent papers (Deng et al., 2017; Mandal et al., 2018), but the role of ROR receptor in RA pathway is not clear.

RA signaling in spermiogenesis and spermiation

RA signal is sufficient for the initiation of meiosis, but not for its completion (Sanjo et al., 2018). However, it still has its role at a later step. Although RA originated from SCs is no longer necessary for subsequent spermatogenic cycles, it is essential for spermiation (Raverdeau et al., 2012).

RAR/RXR

RT-PCR analysis showed that RA stimulated the expression of Stra8 in spermatogonia, but decreased the expression of Nanos2. In the presence of RA, genes involved in postmeiotic development, Tnp1 and Prm1, are up-regulated. The addition of RA receptor (RAR) inhibitor BMS439 indicated that RA enhanced the expression of cAMP response element binding protein through RAR and promoted the formation of round sperm cells (Yu et al., 2019). GCNF could be found in the nuclei of the principal, apical, narrow, clear and halo cells in epididymis (Zhou et al., 2004), and it plays an important role in spermatogenesis, capacitation and fertilization (Xu et al., 2004). GCNF also plays an important role in the regulation of gene expression in early embryo and spermatogenesis with RTR (Lei et al., 1997). A new member of the nuclear receptor superfamily, retinoid receptor-related testis-associated receptor (RTR), was identified and cloned from mouse testis. It was mainly expressed in testis, but not in early germ cells and Sertoli cells, but was most abundant in round sperm cells. This putative transcription factor plays a role in regulating gene expression during the post meiotic stage of spermatogenesis (Hirose et al., 1995). GCNF/RTR may regulate transcription during spermatogenesis, especially in circular spermatozoa, before nuclear elongation and condensation begins (Zhang et al., 1998).

The results of immunohistochemical staining show that RARα and RARγ are mainly located in spermatocytes and round spermatids (but are also present in spermatogonia and Sertoli cells), RARβ is mainly expressed in SCs, and RARγ is expressed in most cell types of human testis. The localization of RARα, RARγ, RXRβ and RXRγ in patients with hypospermatogenesis (HYPO) is similar to that in normal men. In addition, the mRNA expression levels of RARα, RARγ, RXRα, RXRβ and RXRγ are significantly decreased in patients with Sertoli cell syndrome (SCOs) and maturation arrest (MA), but not in patients with hypocytosis. These results suggest that the decrease of RARα, RARγ, RXRα, RXRβ and RXRγ levels is more closely related to the failure of SCOs and MA spermatogenesis (Wang et al., 2020).

RARα plays a role in meiosis, in the transition from round to elongated spermatids, and in SCs of developing testis (Akmal et al., 1997).

Other factors

Stra8 and Setd8 may regulate spermatogenesis in a PCNA-dependent manner through Cdl4-Clu4A-Ddb1 ubiquitinated degradation axis (Niu et al., 2020). ATRA can induce the activities of superoxide dismutase (SOD) and glutathione transferase (GST) in both varicocele and healthy sperm, and reduce the production of malondialdehyde and reactive oxygen species (ROS), thus protecting spermatogenesis (Malivindi et al., 2018). In normal human testis, TSPO exists in interstitial cells and discrete spermatogenesis stage, such as the formation of acrosome of round spermatids, and it can be reduced by RA (Manku and Culty, 2016). miRNAs also play an important role in spermatogenesis. RA can decrease the expression of miR-34c (Li et al., 2013).

Potential clinical application of RA signaling

RA can effectively reverse testicular injury and restore spermatogenesis. It is reported that consumption of a VA-deficient (VAD) diet led to critical defects in spermatogenesis progression (like sperm-head abnormalities (Yokota et al., 2021)), and altered the dynamics of BTB assembly (Chihara et al., 2013), but infertile VAD animals can regain active sperm cells after receiving ROL or RA daily (Huang et al., 1983; Doyle et al., 2009; Nourashrafeddin, 2015). A single injection of all-trans retinoic acid (ATRA) reinitiated spermatogenesis, and inhibition of the function of RA-degrading enzyme CYP26B1 for 10 days induced spermatogonial differentiation in testis of reproductively dormant animals (*Ochotona curzoniae*) (Wang et al., 2019b).

RA can also be used to promote the potential induction of SSC *in vitro* (Li et al., 2014), which is called *in vitro* germ cell induction technology. This new technology may provide a therapeutic strategy for male infertility (Yu et al., 2019). Besides, *in vitro* spermatogenesis with chemically defined media (CDM) involving RA may provide a unique experimental system for research on spermatogenesis that cannot be performed in in vivo experiments (Sanjo et al., 2020).

As RA signaling plays an important role in spermatogenesis, a protein being able to cut off the signaling is considered a viable drug target for male contraceptive development. The testicular lesions produced by treatment with Ro 23-2895 were similar to vitamin A deficiency (Bosakowski et al., 1991), which supports the hypothesis that high doses of synthetic retinoids may cause testicular degeneration through interference of normal retinol homeostasis. High systemic doses of aromatic retinoid (ro10-9359) clearly induce impairment of spermatogenesis, however, all changes were reversible within 6 weeks after withdrawal of the drug (Tsambaos et al., 1980). BDADs can develop safe and effective new contraceptives. WIN18446 treatment of neonatal mice also blocked spermatogonial differentiation and, followed by injection of RA, induced synchronous spermatogenesis in adulthood. The final result is that the sperm is released from the seminiferous epithelium in a pulsatile rather than a normal continuous release (Hogarth et al., 2013). Besides, WIN18446 is also used as a WIN treatment to reduce the tissue concentration of RA by inhibiting alcohol dehydrogenase activity in order to improve post-transplantation repopulation efficiency (Amory et al., 2011). The successful implementation would open up a broader range of application of SSC transplantation technology (Nakamura et al., 2021), from restoration of fertility in young male individuals with cancer following therapy (Firlej et al., 2012) to preservation of genetic diversity of farm animals or endangered species (Honaramooz and Yang, 2010).

Retinoic acid functions via binding to a family of RARs. BMS-189453, an oral RAR pan-antagonist, can lead to marked testicular degeneration and infertility (Schulze et al., 2001; Chung et al., 2011, 2016).

Conclusions and perspectives

As one of the derivatives of Vitamin A, RA regulates the proliferation and differentiation of spermatogonia, meiosis, spermiogenesis and spermiation. As we have discussed above, there are several factors and potential downstream transcriptional regulators important for each step of spermatogenesis. As we can see, GDNF, DMRT and FGF are the most important factors at the self-renewal stage; Stra8, Kit and Nanos are the main factors of RA in differentiation and meiosis. In the spermiogenesis stage, RAR α is a key regulator. Accordingly, these factors have been studied thoroughly. However, there is no specific study on how RA signaling regulates spermiation. In addition, there are still some unsolved problems. For example, DMRT1 regulates both mitosis and meiosis in spermatogenesis, so how does DMRT1 switch between these two stages? Some new regulators have also been found, such as TR2/4 and ROR. Previous studies have found that RA may play a more extensive regulatory role by activating orphan receptors such as TR4 and ROR, but the investigation of this pathway is not complete and needs further study.

Acknowledgements. Our appreciation goes to all members of the Sperm Laboratory in Zhejiang University for their valuable discussions on this study. Dr. Hans-Uwe Dahms is acknowledged for his critical reading of the final draft of the MS.

Authors' roles. H.-Z.Z., and W.-X.Y. wrote the manuscript, S.-L H prepared the references and revision of the manuscript, W.-X.Y. and S.-L H provided the funding.

Funding Information. This project was supported in part by the National Natural Science Foundation of China (No. 32072954 and 32102786).

Conflict of Interest. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Accepted June 6, 2022