The Role of Retinoic Acid (RA) in Spermatogonial Differentiation 1

Authors: Busada, Jonathan T., and Geyer, Christopher B.

Source: Biology of Reproduction, 94(1)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.115.135145

BIOLOGY OF REPRODUCTION (2016) **94**(1):10, 1–10
Published online before print 11 November 2015.
DOI 10.1095/biolreprod.115.135145

Minireview

The Role of Retinoic Acid (RA) in Spermatogonial Differentiation[1]

Jonathan T. Busada[3] and Christopher B. Geyer[2,3,4]

[3]*Department of Anatomy and Cell Biology, Brody School of Medicine, East Carolina University, Greenville, North Carolina*
[4]*East Carolina Diabetes and Obesity Institute, Brody School of Medicine, East Carolina University, Greenville, North Carolina*

ABSTRACT

Retinoic acid (RA) directs the sequential, but distinct, programs of spermatogonial differentiation and meiotic differentiation that are both essential for the generation of functional spermatozoa. These processes are functionally and temporally decoupled, as they occur in distinct cell types that arise over a week apart, both in the neonatal and adult testis. However, our understanding is limited in terms of what cellular and molecular changes occur downstream of RA exposure that prepare differentiating spermatogonia for meiotic initiation. In this review, we describe the process of spermatogonial differentiation and summarize the current state of knowledge regarding RA signaling in spermatogonia.

developmental biology, differentiation, gonocyte, prospermatogonia, retinoic acid, retinoids, spermatogenesis, spermatogonia, testis

INTRODUCTION

Multicellular organisms contain a wide variety of specialized cell types that originate from less specialized cells by cellular differentiation, which involves a progression of specific changes that prepare them for their ultimate function. Many specialized cells have a finite lifespan and therefore must be periodically replaced by a population of uni- or multipotent adult stem cells. These stem cells balance self-renewal with the production of progenitor cells that proliferate to amplify their numbers before committing to a specific cell fate. As an example, the consistent daily production of 10^{12} blood cells in the adult mammalian bone marrow is accomplished by a comparatively small population of hematopoietic stem cells (estimates range from approximately 16 800 to 81 000), the progenitors of which follow unique programs of differentiation to become leukocytes, erythrocytes, or megakaryocytes [1–3].

The production of mammalian spermatozoa in the testis is a stem cell-based developmental process. Each adult mouse testis contains approximately 3000 unipotent spermatogonial stem cells (SSCs) that either self-renew or initiate spermatogenesis by producing undifferentiated progenitor spermatogonia that are destined to enter meiosis [4, 5]. This small population of SSCs is responsible for the production of 10^9 sperm per day throughout the male mouse reproductive lifespan [6]. The decision to remain a stem cell or to proliferate and differentiate is crucial for the reproductive health of the male. In humans, insufficient or excessive differentiation can result in reduced or lost sperm production (nonobstructive oligo- or azoospermia), which are leading causes of male infertility. Strikingly, there appears to be a decrease in overall male reproductive fitness in Western societies over the past several decades, which has been termed testicular dysgenesis syndrome. This syndrome is thought to result from environmental changes and is characterized by a decline in semen quality, increases in hypospadias and cryptorchidism, and an increase in the incidence of testicular cancer [7–9]. Undifferentiated male germ cells (specifically, primordial germ cells, prospermatogonia, and potentially, spermatogonia) that fail to properly differentiate are hypothesized to be the basis for carcinoma in situ, the precursor to most forms of testicular cancer [10–12]. Significant effort has been exerted to understand how the foundational SSC population is maintained, and a number of excellent recent reviews document this progress [13–18]. In contrast, little is known about the cellular changes accompanying spermatogonial differentiation, and the pathways and proteins involved remain poorly defined. The purpose of this review is to provide a developmental perspective on the current state of knowledge about the essential program of spermatogonial differentiation that prepares undifferentiated progenitor spermatogonia for entry into meiosis.

SPERMATOGONIAL BIOLOGY

Following sex determination in the fetal mouse testis, prospermatogonia (also termed gonocytes) proliferate until approximately Embryonic Day (E) 14.5 and then enter a mitotically quiescent state in G_0 of the cell cycle until after birth [19, 20]. Prospermatogonia then re-enter the cell cycle at approximately Postnatal Day (P) 1–2 and migrate from the center to the periphery of the testis cords; completion of both tasks is apparently required for their survival [21]. Prospermatogonia become spermatogonia at approximately P3–P4, as they migrate to the periphery of the testis cords and become flanked by somatic Sertoli cells within the testis cord and

[1]Supported by a grant from the NIH/NICHD (HD072552 to C.B.G.).
[2]Correspondence: Christopher B. Geyer, Brody School of Medicine at East Carolina University, 600 Moye Blvd., Greenville, NC 27834. E-mail: geyerc@ecu.edu

Received: 1 September 2015.
First decision: 29 September 2015.
Accepted: 6 November 2015.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

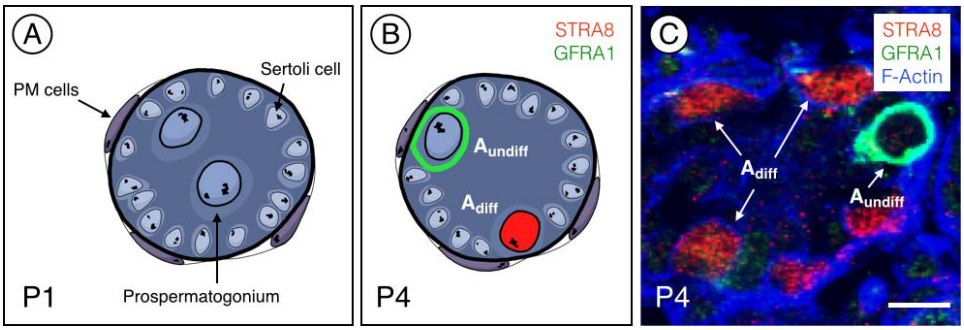

FIG. 1. Germ cell differentiation in the neonatal testis. **A**) At P1, prospermatogonia are located in the center of the testis cords with adjacent Sertoli cells and peritubular myoid (PM) cells surrounding the tubules. **B** and **C**) By P4, spermatogonia have become either A_{undiff} (GFRA1+, in green) or A_{diff} (STRA8+, in red). In **C**, the testis cords are outlined by F-actin staining with phalloidin (in blue). Bar = 10 μm.

peritubular myoid cells that surround the outside of the cord (Fig. 1, A and B). The proteins and signaling networks involved in this transition are currently under investigation by several laboratories. It has recently been shown that suppressing NOTCH signaling in Sertoli cells is important for maintaining quiescence in prospermatogonia [22–24]. Also, two reports indicate the requirement for the chromatin-modifying protein Swi-independent 3a (SIN3A) in regulation of mitotic re-entry [25, 26]. Members of the transforming growth factor beta (TGF-β) superfamily, such as the activins, inhibins, and bone morphogenetic proteins (BMPs), have likely roles in the initiation of spermatogenesis, although their requirement in vivo requires further study (for reviewed, see [27]).

This initial neonatal spermatogonial population is heterogeneous, and both undifferentiated (A_{undiff}) and differentiating (A_{diff}) spermatogonia are detectable as early as P3–P4 (Fig. 1C) [28–31]. The origin of this heterogeneity is currently undefined, although it is apparent that spermatogonia are capable of differentially responding to extrinsic signals from somatic cells, which will be discussed in greater detail below. A small percentage of the A_{undiff} population contains the future SSC pool, which functions to support steady-state spermatogenesis throughout the remainder of the male reproductive lifespan. The rest of the surviving neonatal spermatogonia become progenitor or differentiating spermatogonia that will enter meiosis beginning at approximately P10 to give rise to the first fertilizable sperm that are seen around P35. It is presumed that the first differentiating spermatogonia arise directly from prospermatogonia without first forming an SSC [29, 30, 32]. This transition (prospermatogonia to type A spermatogonia) marks the ''initiation of spermatogenesis'' in the mouse.

During steady-state spermatogenesis, the products of SSC divisions either maintain the stem cell pool (self-renewal division) or generate progenitor spermatogonia that will proliferate and differentiate to eventually enter meiosis. Daughter cells of an SSC division that are destined to differentiate retain a relatively large (~1 μm diameter) tubular connection, termed an intercellular or cytoplasmic bridge, that results from incomplete cytokinesis [33, 34]. The function of these bridges is unclear. However, they are highly conserved through evolution and allow sharing of molecules and even organelles such as mitochondria between cells within a syncytium (for review, see [35]). This likely aids in the synchronization of subsequent divisions, and it may also provide for the sharing of essential X-linked gene products between adjacent X- and Y-bearing haploid postmeiotic spermatids later during spermiogenesis. Single spermatogonia are termed A_{single} (A_s), whereas those connected by an intercellular bridge are termed A_{paired} (A_{pr}). As progenitor spermatogonia undergo transit-amplifying divisions, they form progressively longer interconnected chains as $A_{aligned}$ (A_{al}) spermatogonia. Although A_s, A_{pr}, and A_{al} spermatogonia are all classified as undifferentiated, evidence supports a diminution of stem cell capacity that accompanies increased chain length (for review, see [17]). However, evidence indicates that some A_{pr} spermatogonia, termed false pairs, represent SSCs that have divided but not moved away from one another (for review, see [13]).

The commitment to enter meiosis is made with the transition of A_{undiff} into A_{diff} spermatogonia. This transition requires retinoic acid (RA), which will be discussed in detail below. The first differentiating spermatogonia are termed type A_1, which undergo five subsequent divisions to form A_2, A_3, A_4, intermediate (In), and finally, type B spermatogonia before becoming preleptotene spermatocytes that are in the first phase of meiosis I. During differentiation, the cell-cycle duration decreases, and a significant amount of germ cell loss occurs, such that only an estimated 39% of the expected numbers of preleptotene spermatocytes are formed [36–38]. It is important to note that male germ cells must complete this prolonged stepwise differentiation process that takes approximately 1 wk in order to gain competence to enter and successfully complete meiosis. In the fetal testis, prospermatogonia respond to precocious RA exposure by inappropriately expressing meiotic markers before rapidly dying by apoptosis [39–41]. In the neonatal and adult testis, A_{undiff} spermatogonia exposed to exogenous RA cannot be hastened to enter meiosis without progressing through these steps [42–46].

The accurate identification of spermatogonial subtypes at the histological level takes a considerable amount of experience, and it relies on characteristic differences in nuclear shape and diameter as well as heterochromatin appearance in paraffin-embedded testis sections carefully prepared with certain fixatives (e.g., 5% glutaraldehyde or Bouin solution). In the adult testis, identification is aided by the fact that germ cells are present in defined stages of the seminiferous epithelium [29, 47]. Neonatal and juvenile testes lack clearly defined epithelial stages, although some have proposed that staging is possible beginning with the appearance of preleptotene spermatocytes at approximately P8. The absence of defined stages makes it difficult to impossible to discriminate reliably between all spermatogonial subtypes at the light microscopic level based on morphology alone. This is because differences in morphology (especially of chromatin) are both subtle and quite variable [28, 48]. The topological arrangement of A_s, A_{pr}, and A_{al} spermatogonia can be visualized in whole

	Neonate		Adult	
	A_{undiff}	A_{diff}	A_{undiff}	A_{diff}
ID4	+	-	+	-
GFRA1	+	-	+	-
RET	+	-	+	-
ZBTB16/PLZF	+	+	+	-
CDH1	+	+	+	-
PAX7	+	-	+	-
NANOS2*	+	-	+	-
NANOS3*	+	-	+	-
KIT	-	+	-	+
STRA8	-	+	-	+
RHOX13	+	+++	+	+++
SOHLH1	faint	++	-	+
SOHLH2	faint	++	-	+

FIG. 2. Selected spermatogonial protein fate markers. The estimated detection level for each marker is listed by a plus sign (+, ++, or +++). Levels are subjective, and comparisons are only valid for tissues prepared, incubated, and stained similarly. The minus sign (–) indicates that the listed protein is undetectable but not necessarily absent from the listed cell type. *Inferred (largely studies using fluorescent reporter constructs in transgenic mice).

mounts of seminiferous cords or tubules, but the germ cells must be labeled either by transgenic expression of a fluorescent reporter such as GFP or by using indirect immunofluorescent antibody staining, as described below.

Another useful tool for spermatogonial identification is labeling with antibodies against specific protein markers that have been linked to cell fate or function (Fig. 2). A number of characteristic proteins have been identified that are detectable primarily in A_{undiff} spermatogonia in the adult testis, including ID4, GFRA1, RET, ZBTB16/PLZF, CDH1 and PAX7 [17] as well as NANOS2 and NANOS3 (which are indirectly labeled as FLAG-tagged transgenes) [49, 50]. In contrast, KIT and STRA8 are the only two markers currently detectable in differentiating, but not undifferentiated, spermatogonia [51–53]. Although STRA8 is generally considered to be a nuclear protein, it has also been detected in the cytoplasm [54, 55]. Its function is currently unknown, but its determination should help clarify this observation. For A_{diff} subtypes (A_1, A_2, A_3, or A_4), no protein markers are currently identified that allow them to be distinguished from one another without knowing the stage of the adult seminiferous tubule within which they reside. Some protein markers have increased levels in differentiating spermatogonia, including RHOX13, SOHLH1, and SOHLH2 [42, 56–60]. Whereas the above markers are restricted to either undifferentiated or differentiating spermatogonia in the juvenile and adult testis, this is largely not true in the neonatal testis. We recently reported significant overlap, in that markers for A_{undiff} colocalized with the spermatogonial differentiation marker KIT (as well as STRA8) through approximately P10, with GFRA1 and RET being notable exceptions [44]. This suggests that the differentiating program is overlaid on the undifferentiated state in prospermatogonia and spermatogonia in the neonatal testis. Whereas these fates appear more clearly established in the juvenile and adult, transient overlap of some undifferentiated and differentiating markers also occurs in adult spermatogonia.

Three types of spermatogonia are present in the human testis—namely, types A_{dark}, A_{pale}, and B. Prospermatogonia transition into both A_{dark} and A_{pale} spermatogonia by 2–3 mo after birth. The first differentiating type B spermatogonia are visible by 4–5 yr of age but only represent approximately 10% of the spermatogonial population by age 10 [61]. It is currently held that A_{dark} spermatogonia are non- or slow-cycling and represent the ''reserve'' stem cell population that can be activated following damage to the germ cell population. In contrast, A_{pale} spermatogonia are analogous to the A_{undiff} population in rodents and can be present as single cells or longer chains of interconnected cells (for review, see [62]).

EXTRINSIC SIGNALS DIRECT SPERMATOGONIAL FATE

Tissue homeostasis is maintained in many epithelial tissues that have a high rate of turnover through a delicate balance between stem cell self-renewal and the production of progenitors that proliferate and differentiate. In the testis, distinct signals received by developing male germ cells direct this balance. Numerous studies have revealed that the undifferentiated state is maintained by the binding of the ligands provided by Sertoli and/or peritubular myoid cells to their cognate receptors on spermatogonia both in vivo and in vitro. While numerous additional signaling pathways surely await discovery, the best described ligand/receptor pairs currently include glial cell-derived neurotrophic factor (GDNF) to GFRA1/RET [63–70], chemokine (C-X-C motif) ligand 12 (CXCL12) to CXCR4 [71], and fibroblast growth factors 2 and 8 (FGF2 and FGF8, respectively) to FGFR [72–77].

Spermatogonial differentiation requires all-*trans* retinoic acid (ATRA; referred to as RA), the bioactive oxidative metabolite of vitamin A/retinol [46, 78–81]. Spermatogonia cannot progress past the A_{al} stage in mice when RA signaling is blocked following prolonged consumption of a vitamin A-deficient (VAD) diet or administration of specific compounds (e.g., bis-[dichloroacetyl]-diamines such as WIN 18 446) that restrict the synthesis of RA from retinal by inhibiting retinaldehyde dehydrogenases [82–84]. Either treatment causes arrested spermatogonial differentiation at the A_{al}-to-A_1 transition that can be reversed by retinoid supplementation, resulting in resumption of spermatogenesis and restoration of fertility [79, 82, 83, 85]. Although the primary role for RA in the testis in directing spermatogonial differentiation is clearly established, the mechanisms activated downstream of RA exposure are largely undefined.

REGULATING SPERMATOGONIAL EXPOSURE TO RA

Cellular exposure to RA is managed at multiple levels by proteins that regulate its synthesis, reception, storage/transport, and degradation [85–87]. Several laboratories are currently focused on understanding how RA is distributed within the testis such that only A_{diff} spermatogonia respond to this differentiating signal. Two general scenarios can be envisioned. In option 1, all spermatogonia are primed to respond to RA, but the exposure to RA is tightly controlled; in option 2, all spermatogonia are exposed to RA, but only some can respond. Current evidence in the literature suggests that both scenarios are involved (see Fig. 3A). In support of this notion, the postnatal deletion of single, seemingly key molecules involved in RA reception, storage, and degradation in knockout (KO) mouse models has not thus far resulted in phenotypes that fully recapitulate the VAD model's arrested spermatogonial differentiation and infertility [88–96]. This indicates that both exposure and reception are parts of a complementary system

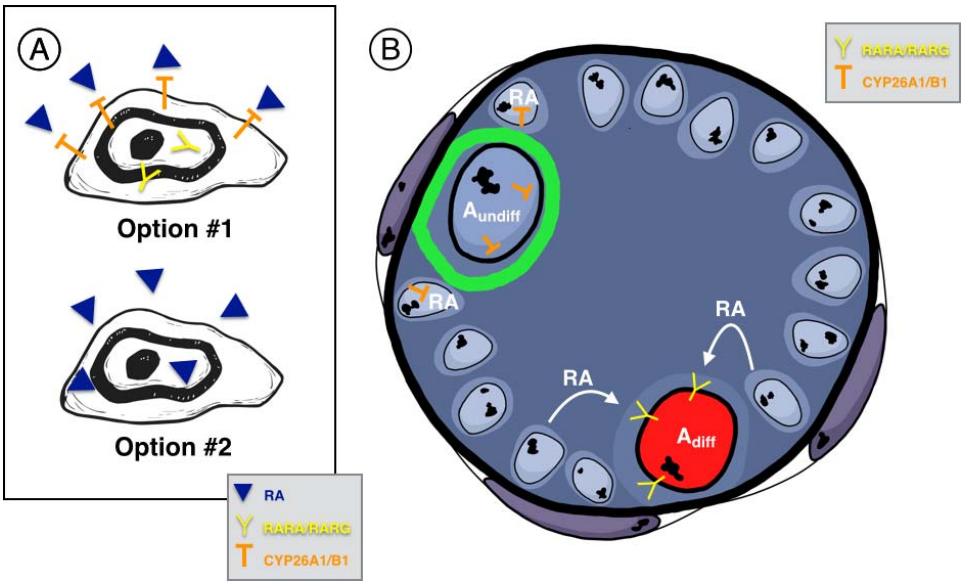

FIG. 3. Regulating spermatogonial exposure to RA. **A**) Two options, described in the text, for how spermatogonia become exposed to or avoid RA. In option 1, an SSC contains RARs (yellow Y) and so could presumably differentiate in response to RA (blue triangle). However, expressed CYP26 (orange T) catabolizes RA and prevents binding to RARs. In option 2, an SSC that does not express RARs is pictured. Therefore, although the cell has access to abundant RA, it does not differentiate because it lacks the requisite RARs to transduce the signal. **B**) Two type A spermatogonia within the same cord adopt different fates based on their response to RA. The upper left spermatogonium remains GFRA1+ (green) and can respond to GDNF but does not respond to RA. This may result from lack of requisite RARs and/or degradation by CYP26 enzymes active in either the spermatogonium itself or in the RA-producing cell (adjacent Sertoli). The lower right spermatogonium responds to RA by becoming STRA8+ (red) and KIT+ (not shown) and differentiating. This may result from gain of RAR expression or the absence of localized CYP26-mediated degradation within the spermatogonium itself or adjacent Sertoli cells.

with redundant controls built in to ensure spermatogonia respond appropriately to RA.

Results from several reports support a role for regulated RA exposure (option 1 above) in maintaining spermatogonial cell fate. In the fetal testis, quiescent prospermatogonia must be protected from RA exposure or they will begin to differentiate and enter meiosis precociously and, as a result, die by apoptosis [40, 41]. This protection is provided, at least in part, by the RA-degrading action of the cytochrome P450 enzyme CYP26B1 [39, 94]. After birth, a subset of spermatogonia becomes exposed to RA by P3–P4 (as evidenced by their expression of the RA-inducible *Stra8* gene) [42, 53, 58, 84, 97]. If CYP26B1-mediated degradation is responsible for protecting a subset of postnatal spermatogonia from RA exposure, this implies that degradation activity is reduced or lost near STRA8+ A_{diff} spermatogonia, although this has not been shown experimentally. In the adult testis, the majority of A_{undiff} spermatogonia transition to differentiating KIT+ A_1 spermatogonia at stage VIII of the seminiferous epithelial cycle. This coincides with STRA8 induction in A_{diff} spermatogonia and preleptotene spermatocytes [54, 98], and it was recently shown that a pulse of RA peaks at stage VIII [51]. Therefore, RA levels are clearly modulated during steady-state spermatogenesis in the adult; epithelial stages VII–VIII, which are exposed to the highest levels of RA, contain germ cells undergoing the three processes that are dependent upon RA (spermatogonial differentiation, meiotic initiation, and spermiation) [46, 79, 99, 100].

Evidence supports a requirement for the production of RA by Sertoli cells. Circulating retinol is converted into RA by two successive reactions: retinol to retinal by retinol dehydrogenases, and retinal to RA by retinaldehyde dehydrogenases. The conditional deletion of retinol dehydrogenase 10 (*Rdh10*) in Sertoli plus germ cells, and, to a lesser extent, in Sertoli cells only, resulted in loss of A_1 differentiating spermatogonia [101]. Interestingly, this defect is only manifest during the first wave of spermatogenesis in young mice (age, <7 wk); after that, KO males exhibit normal fertility and testis histology. These results clearly suggest that the first step in the synthesis of RA (retinol to retinal) is performed by another retinol dehydrogenase during steady-state spermatogenesis in the adult. In addition, three retinaldehyde dehydrogenases (*Aldh1a1–3*, previously termed *Raldh1–3*) have been conditionally deleted in mouse Sertoli cells [45]. Spermatogonia in these mice fail to differentiate; however, injection of RA or a retinoic acid receptor (RAR) A-selective agonist reinitiates spermatogenesis.

There is also evidence supporting a role for regulated RA reception (option 2 above) in maintaining spermatogonial cell fate. RA is a lipid-soluble molecule that enters the cell to bind with high affinity to its cognate receptors. The RAR has three isotypes (RARA, RARB, and RARG), and each is capable of heterodimerizing with a retinoid X receptor isotype (RXRA, RXRB, or RXRG). The RAR isotypes have distinct expression patterns in the testis: RARB is undetectable, RARA predominates in Sertoli cells, and RARG predominates in A_{diff} spermatogonia in the neonatal, juvenile, and adult testis [92, 102]. Therefore, based on this expression pattern, it is logical to assume that RA signaling through RARG directs spermatogonial differentiation. Whole-body as well as germ cell and Sertoli cell KO mice have been generated with deletions of each of the RAR isotypes to address their respective roles in the testis. *Rarb*-null mice are viable and fertile, with no apparent defects in spermatogenesis [103], which is expected based on its apparent lack of expression in the testis. In contrast, both *Rara*-null and *Rarg*-null mice exhibit varying defects in spermatogenesis, although loss of either gene singly or in combination does not recapitulate the VAD phenotype of blocked spermatogonial differentiation [88–90, 92, 104, 105]. Although *Rara* KO mice are infertile [88, 89, 105], deletion of *Rarg* has no obvious effect on spermatogonial differentiation during the first wave of spermatogenesis, and many tubules are apparently normal until KO mice reach advanced age (~12

mo) [92]. Unfortunately, the reproductive performance of *Rarg* germ cell KO males has not been reported; based on the histology images provided in Gely-Pernot et al. [92], it is reasonable to expect that young KO mice are fertile. Therefore, it can be concluded that although RAR isotypes clearly participate in spermatogonial differentiation, none is essential for the process to occur. As mentioned above, seminiferous epithelial stages VII–VIII are exposed to the highest measured RA levels [22, 43, 51], and spermatogonia differentiate during this time. However, stage-VII and -VIII tubules also contain SSCs, which remain undifferentiated and STRA8−/KIT−, implying that they did not receive the RA signal. A recent study employed a transgenic approach to create mice expressing RARG from the *Gfra1* promoter in A_{undiff} spermatogonia; the results indicate that these spermatogonia inappropriately differentiate during stages VII–VIII [102]. These results, taken together with the *Rarg* KO data above, support the concept that RARG is sufficient, but not required, to direct spermatogonial differentiation.

The testis contains a consistently small SSC population and a system for the proliferation and then differentiation of millions of progenitor spermatogonia required for gamete production. Accordingly, it should not be surprising that the responsiveness of spermatogonia to RA is controlled by redundant mechanisms (expression of RARG in spermatogonia primed to differentiate but *not* in SSCs, and careful modulation of RA levels by RA-synthesizing and -degrading enzymes) (see Fig. 3B). The evidence that such a robust system is seemingly not reliant upon the function of a single gene product highlights how critical modulating male germ cell RA exposure is for both male reproductive health and success.

GENE EXPRESSION CHANGES DURING DIFFERENTIATION

Although it has been known for 90 yr that retinoids are essential for male fertility [78], the molecular and cellular events downstream of RA remain largely undefined. A primary reason for our lack of knowledge about spermatogonial differentiation is that studies using whole-genome approaches have identified few changes in steady-state mRNA levels between A_{undiff} and A_{diff} spermatogonia or in whole testes during the early phases of neonatal testis development during which differentiation takes place [98, 106, 107]. Many of the upregulated spermatogonia-expressed genes (at the mRNA level) encode proteins with known roles in meiosis (e.g., REC8, STRA8, and SYCP3) [53, 97, 108–110]. This is not meant to conclude that transcriptional regulation is uninvolved in spermatogonial differentiation. Indeed, the deletion of the transcription factors *Sohlh1*, *Sohlh2*, and *Sox3* blocks (in *Sohlh1* and *Sohlh2* KO testes) or impairs (in *Sox3* KO testes) spermatogonial differentiation, although the defects in *Sox3* germ cell KO mice are more severe during the first wave of spermatogenesis and improve over time as the mice age [56, 111–114]. In addition, testis cords are apparently normal through at least P10, which argues against an absolute requirement for SOX3 during spermatogonial differentiation.

It has become clear through genomic and genetic analyses that genes required to maintain SSCs in vivo and in vitro are lost in response to spermatogonial differentiation signals [14–17, 115]. One example is *Zbtb16*/*Plzf*, which encodes a putative transcriptional repressor that is detectable in A_{undiff} spermatogonia in the adult but is present in all spermatogonia in the neonatal and juvenile testis [44, 116, 117]. ZBTB16 is not absolutely required for spermatogenesis; although adult mutant and KO mice are infertile, with very low numbers of epididymal sperm, their testes contain numerous Sertoli cell-only tubules (lacking germ cells) adjacent to rather normal-appearing tubules [116, 117]. A role in SSC self-renewal is concluded based on the histological phenotype and because mutant spermatogonia are unable to colonize testes of recipient mice lacking germ cells [116, 117].

Without dramatic upregulation of mRNAs during spermatogonial differentiation, scientists have lacked targets in the form of proteins and pathways for focused studies. The lack of dramatic differences in the transcriptome of developing spermatogonia suggests, first, very few changes in transcription or mRNA decay (both contribute to resulting steady-state mRNA levels) or, second, differences in the transcriptomes within spermatogonial subtypes that are not discernible when an entire population of spermatogonia is queried. Results from a recent study suggest that both are viable options. Significant differences exist in the abundance of specific mRNAs within single spermatogonia that correlate with their fate status (expression of *Id4*-GFP in SSCs) [118]. This work also highlights examples of genes for which the mRNAs are present in *Id4*-GFP+ SSCs without detectable protein, which indicates posttranscriptional regulation of gene expression.

It has been known for many years that not all mRNAs are translated into protein with similar efficiency—mRNAs can be stored, can be inefficiently or efficiently translated, or can be targeted for degradation [119]. The decision among these biochemical fates provides cells with an important level of control over gene expression that allows rapid and large-scale responses to developmental stimuli. Recent results provide a novel perspective that may advance our understanding of events during spermatogonial differentiation. Others and we have reported that mRNAs encoding essential differentiation factors (e.g., KIT, SOHLH1, and SOHLH2) are present, but repressed, in A_{undiff} spermatogonia but then initiate translation in response to RA in A_{diff} without a dramatic increase in mRNA abundance [42, 58, 120, 121]. This suggests that, instead of transcriptional activation of unique genes, efficient translation of a pool of repressed mRNAs is the driving force behind gene expression changes during spermatogonial differentiation. Two well-studied mechanisms for posttranscriptional control of gene expression involve miRNAs and RNA-binding proteins, and evidence is growing that both are involved in spermatogenesis.

MicroRNAs are short, noncoding RNAs that bind to target mRNAs and repress their translation by inducing cleavage or destabilization or by preventing ribosomal association (for review, see [122]). The biogenesis of requires the ribonuclease *Dicer1*. Somewhat surprisingly, deletion of *Dicer1* in fetal male germ cells does not cause noticeable defects until the pachytene stage of meiosis [123–127], indicating that miRNA function is not essential for spermatogonial development. However, recent studies indicate that specific miRNAs and miRNA clusters (*Mir146*, *Mir221/222, Mirc1, Mirc3,* and *Mirlet7*) do contribute to gene regulation in spermatogonia [121, 128–130]. In vivo, however, they likely play a supplementary or supportive role in the translational control of mRNAs encoding factors that direct spermatogonial development. In contrast to the dispensable function of miRNAs in premeiotic male germ cells, numerous RNA-binding proteins have been shown to be essential for fetal and neonatal mammalian germ cell development and differentiation (e.g., NANOS2, NANOS3, DAZL, TIAR/TIAL1, PIWIL2/MILI, PIWIL4/MIWI2, and DDX4/VASA) [131–135]. The most well characterized RNA-binding protein is NANOS2, which is required for the function and survival of both fetal prospermatogonia and postnatal SSCs [50, 136]. It appears to

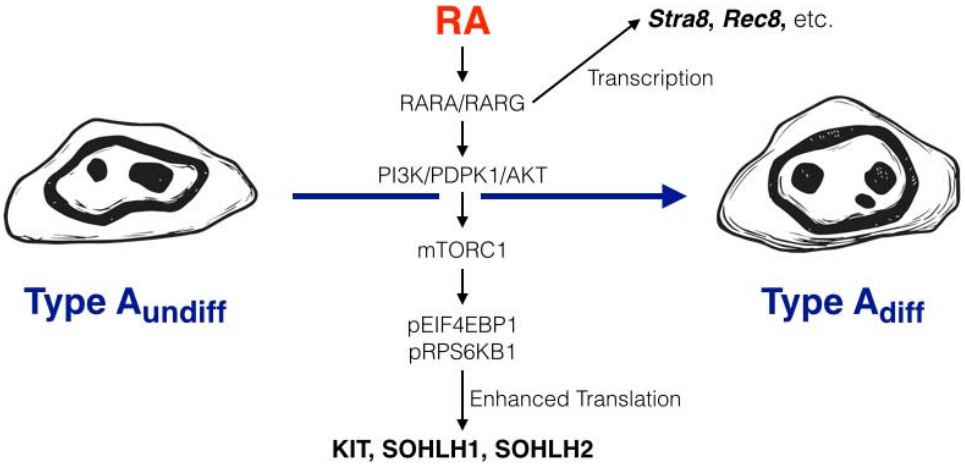

FIG. 4. RA stimulates transcription and PI3K/AKT/mTOR kinase signaling in differentiating spermatogonia. RA transcriptionally activates genes required for meiosis (e.g., *Stra8* and *Rec8*) and enhances the translational efficiency of repressed mRNAs required for spermatogonial differentiation (e.g., *Kit*, *Sohlh1*, and *Sohlh2*) through activation of kinase signaling.

have multiple roles in suppressing translation of target mRNAs during spermatogenesis, both by promoting their degradation [137] and by preventing association with polyribosomes [138]. A formal link between RA and expression of NANOS2 has not been established in the postnatal testis, but in fetal testes of mice lacking *Cyp26b1* (which have higher RA levels), *Nanos2* mRNA levels were significantly reduced [139]. This suggests that RA may negatively regulate the expression of *Nanos2*; indeed, exogenous RA decreases *Nanos2* mRNA levels in the neonatal testis (our unpublished data). The loss of NANOS2 expression downstream of RA signaling provides a potential mechanistic explanation for the increased translational efficiency of repressed mRNAs encoding determinants of spermatogonial differentiation such as KIT, SOHLH1, and SOHLH2.

Additional evidence supporting the importance of regulated protein synthesis during spermatogonial development comes from studies of mutant and KO mice. The first is from studies using ''juvenile spermatogonial depletion'' (*jsd*) mutant mice, which exhibit a normal first wave of spermatogenesis followed by a complete loss of all germ cells in the adult except for A_{undiff} spermatogonia [140–143]. The mutated gene responsible for this phenotype was later identified as *Utp14b* [141, 142], which is an autosomally encoded, processed retroposon copy of the X-linked *Utp14a* gene required for 18S ribosomal RNA processing during ribosome biogenesis. Interestingly, raising testicular temperature in adult mice restores spermatogenesis, leading to the creation of fertilizable sperm [144–146]. This suggests that this naturally occurring mutation is temperature-sensitive, which may explain why no phenotype is apparent during the first wave of spermatogenesis, much of which occurs at 37°C. The phenotype of adult mice, which lack UTP14B function, suggests that ribosome biogenesis is an important aspect of spermatogonial proliferation and differentiation but is not critical in A_{undiff} spermatogonia. The second example is provided by mice lacking *Eif2s3y*, a Y-linked gene that encodes a subunit of the eukaryotic initiation factor complex EIF2, which forms a ternary complex with GTP and Met-tRNA during translation initiation. KO testes only contain GFRA1+ A_{undiff} spermatogonia, indicating that EIF2S3Y is required for spermatogonial proliferation and differentiation [147–151]. Coincidentally, *Eif2s3y* is one of the two genes (the other being *Sry*) on the Y chromosome required for male fertility [152]. What specific role this EIF2 gamma subunit isoform plays during spermatogonial proliferation and differentiation is currently unknown, but it is tempting to speculate that it is required for the program of translational activation of repressed mRNAs, as described above.

A perplexing question is why would undifferentiated male germ cells rely on a system of translational control over a subset of mRNAs, as it seems to be a rather inefficient use of cellular resources? This difficult question has many potential answers, and we may never know which one is the most correct. First, transcription is not a particularly expensive process in terms of cellular ATP output, especially in comparison with protein synthesis and degradation [153, 154]. The simplest explanation may be that prospermatogonia and A_{undiff} spermatogonia lack the ability to precisely control transcription of certain genes and therefore employ posttranscriptional controls as a means to regulate gene expression. An example is provided by the KIT receptor tyrosine kinase; whereas the mRNA is detectable throughout testis development, the protein is only present in discrete stages (for review, see [52]). Protein is expressed in primordial germ cells and is required for their proper migration to the developing fetal gonad. After their colonization, KIT protein expression is silenced for over a week in prospermatogonia but is required again beginning at P3–P4 in a subset of type A spermatogonia for their differentiation [52].

KINASE SIGNALING DURING SPERMATOGONIAL DIFFERENTIATION

The best-studied action of RA is genomic, in which RA stimulates transcription by binding RARs on RA response elements (RAREs) in target gene promoters. However, lack of significant changes in steady-state mRNA levels after RA exposure suggests other avenues of RA-based regulation may be utilized in the neonatal testis [98, 107]. Compelling evidence in other systems indicates that RA can also utilize alternative, nongenomic pathways via kinase cascades [155, 156]. For example, RARA is bound to the regulatory subunit (p85) of PI3K in several cell types (SH-SY5Y, NIH3T3, and MEFs). RA addition causes the recruitment of the catalytic subunit (p110) to induce rapid phosphorylation of ERK and AKT [155, 156]. In support of this, our laboratory discovered that RA activates the PI3K/PDK1/AKT/mTORC1 signaling pathway, and this is required for translation of repressed

mRNAs such as *Kit*, *Sohlh1*, and *Sohlh2* [42, 58, 120]. Activation of the PI3K/AKT signaling pathway following binding of the receptor tyrosine kinase KIT by KITL is essential for spermatogonial development [157–160]. It is plausible that a main role for RA in the testis is to initiate the expression of KIT protein in A_{diff} spermatogonia, which then binds KITL to maintain activated PI3K/AKT signaling.

Signaling through PI3K and AKT transmits growth, proliferation, differentiation, and survival signals [161]. These signals can directly impinge upon protein synthesis regulation through mTORC1 activation. Addition of exogenous RA to P1 mice led to an increase in mTOR phosphorylation (and therefore activation) in prospermatogonia [58]. Among the substrates phosphorylated by mTOR are the EIF4E-binding protein EIF4EBP1, which specifically mediates cap-dependent mRNA translation, and ribosomal protein S6-kinase (S6K), which modifies ribosomes directly in conjunction with their enhanced synthetic activity [162, 163]. Thus, phosphorylation of EIF4EBP1 and S6K provide two direct, but distinct, indicators of translational activity in prospermatogonia, as they differ in response to RA.

The importance of kinase signaling in spermatogonial proliferation and differentiation is highlighted by genetic studies in which members or regulators of this signaling pathway are conditionally inactivated with *Ddx4*-Cre, which is first expressed at E15 in fetal prospermatogonia [164]. In the first study, deletion of *Pdk1* (also *Pdpk1*, activator of AKT) or *Foxo1* (downstream target of AKT) results in reduced KIT expression and impaired spermatogonial differentiation [165]. In another study, mTORC1 is precociously activated in A_{undiff} spermatogonia by deleting *Tsc2*, which normally functions to indirectly inhibit mTORC1 activity through RHEB [166]. Results of that study indicate that suppression of mTORC1 is important for SSC maintenance, which implies that mTORC1 activation is required for differentiation. A complementary study from our laboratory supports this, as we found that mTORC1 inhibition by rapamycin blocks spermatogonial differentiation in vivo [120]. Taken together, these results support a model in which RA signals through kinase pathways that in turn activate mTORC1 as a requisite step in spermatogonial differentiation (Fig. 4).

CONCLUSION AND FUTURE DIRECTIONS

Spermatogonia differentiate in response to RA and undergo largely unknown, yet clearly essential, cellular and molecular changes that precede meiosis. A major form of gene regulation during spermatogonial development may be exerted posttranscriptionally downstream of kinase signaling pathway activation. Future work should focus on identifying changes in the proteome and further clarifying the involvement of signaling pathways and the consequences of their actions. These efforts will, in our opinion, prove fruitful in the identification of specific cellular processes during differentiation that prepare spermatogonia for entry into meiosis. In addition, exploring this understudied phase of male germ cell development will have the added benefit of providing critical insights regarding the programs that precede (SSC self-renewal) and follow (meiosis).

ACKNOWLEDGMENT

The authors would like to thank Cathryn Hogarth (Washington State University) and Lance Bridges (East Carolina University) for critically reading the manuscript. Drawings were done by East Carolina University art students Sydney Hinkle and Madalyn McLeod.

REFERENCES

1. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. Cell Stem Cell 2012; 10:120–136.
2. Gordon MY, Lewis JL, Marley SB. Of mice and men. . .and elephants. Blood 2002; 100:4679–4680.
3. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 2008; 132:631–644.
4. Nagano MC. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 2003; 69: 701–707.
5. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 2000; 97:8346–8351.
6. Kyjovska ZO, Boisen AM, Jackson P, Wallin H, Vogel U, Hougaard KS. Daily sperm production: application in studies of prenatal exposure to nanoparticles in mice. Reprod Toxicol 2013; 36:88–97.
7. Andersson AM, Jorgensen N, Main KM, Toppari J, Rajpert-De Meyts E, Leffers H, Juul A, Jensen TK, Skakkebaek NE. Adverse trends in male reproductive health: we may have reached a crucial ''tipping point.'' Int J Androl 2008; 31:74–80.
8. Jensen TK, Sobotka T, Hansen MA, Pedersen AT, Lutz W, Skakkebaek NE. Declining trends in conception rates in recent birth cohorts of native Danish women: a possible role of deteriorating male reproductive health. Int J Androl 2008; 31:81–92.
9. Skakkebaek NE, Jorgensen N, Main KM, Rajpert-De Meyts E, Leffers H, Andersson AM, Juul A, Carlsen E, Mortensen GK, Jensen TK, Toppari J. Is human fecundity declining? Int J Androl 2006; 29:2–11.
10. Sharpe RM, Skakkebaek NE. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil Steril 2008; 89:e33–e38.
11. Skakkebaek NE. Possible carcinoma-in-situ of the testis. Lancet 1972; 300:516–517.
12. Sonne SB, Kristensen DM, Novotny GW, Olesen IA, Nielsen JE, Skakkebaek NE, Rajpert-De Meyts E, Leffers H. Testicular dysgenesis syndrome and the origin of carcinoma in situ testis. Int J Androl 2008; 31:275–287.
13. de Rooij DG, Griswold MD. Questions about spermatogonia posed and answered since 2000. J Androl 2012; 33:1085–1095.
14. Kanatsu-Shinohara M, Shinohara T. Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol 2013; 29:163–187.
15. Nagano MC, Yeh JR. The identity and fate decision control of spermatogonial stem cells: where is the point of no return? Curr Top Dev Biol 2013; 102:61–95.
16. Oatley JM, Brinster RL. The germline stem cell niche unit in mammalian testes. Physiol Rev 2012; 92:577–595.
17. Yang QE, Oatley JM. Spermatogonial stem cell functions in physiological and pathological conditions. Curr Top Dev Biol 2014; 107:235–267.
18. Yoshida S. Stem cells in mammalian spermatogenesis. Dev Growth Differ 2010; 52:311–317.
19. Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG. Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 1991; 93:233–243.
20. Western P, Miles D, van den Bergen J, Burton M, Sinclair A. Dynamic regulation of mitotic arrest in fetal male germ cells. Stem Cells 2008; 26: 339–347.
21. Roosen-Runge EC, Leik J. Gonocyte degeneration in the postnatal male rat. Am J Anat 1968; 122:275–299.
22. Garcia TX, DeFalco T, Capel B, Hofmann MC. Constitutive activation of NOTCH1 signaling in Sertoli cells causes gonocyte exit from quiescence. Dev Biol 2013; 377:188–201.
23. Garcia TX, Farmaha JK, Kow S, Hofmann MC. RBPJ in mouse Sertoli cells is required for proper regulation of the testis stem cell niche. Development 2014; 141:4468–4478.
24. Garcia TX, Hofmann MC. NOTCH signaling in Sertoli cells regulates gonocyte fate. Cell Cycle 2013; 12:2538–2545.
25. Gallagher SJ, Kofman AE, Huszar JM, Dannenberg JH, DePinho RA, Braun RE, Payne CJ. Distinct requirements for *Sin3a* in perinatal male gonocytes and differentiating spermatogonia. Dev Biol 2013; 373:83–94.
26. Pellegrino J, Castrillon DH, David G. Chromatin associated Sin3A is essential for male germ cell lineage in the mouse. Dev Biol 2012; 369: 349–355.
27. Itman C, Wong C, Whiley PA, Fernando D, Loveland KL. TGFβ superfamily signaling regulators are differentially expressed in the developing and adult mouse testis. Spermatogenesis 2011; 1:63–72.
28. Drumond AL, Meistrich ML, Chiarini-Garcia H. Spermatogonial

morphology and kinetics during testis development in mice: a high-resolution light microscopy approach. Reproduction 2011; 142:145–155.
29. Kluin PM, de Rooij DG. A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. Int J Androl 1981; 4:475–493.
30. Kluin PM, Kramer MF, de Rooij DG. Proliferation of spermatogonia and Sertoli cells in maturing mice. Anat Embryol (Berl) 1984; 169:73–78.
31. Nagano R, Tabata S, Nakanishi Y, Ohsako S, Kurohmaru M, Hayashi Y. Reproliferation and relocation of mouse male germ cells (gonocytes) during prespermatogenesis. Anat Rec 2000; 258:210–220.
32. Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, Nabeshima Y. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 2006; 133:1495–1505.
33. Dym M, Fawcett DW. Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. Biol Reprod 1971; 4:195–215.
34. Weber JE, Russell LD. A study of intercellular bridges during spermatogenesis in the rat. Am J Anat 1987; 180:1–24.
35. Greenbaum MP, Iwamori T, Buchold GM, Matzuk MM. Germ cell intercellular bridges. Cold Spring Harb Perspect Biol 2011; 3:a005850.
36. Huckins C. The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. Cell Tissue Kinet 1971; 4:313–334.
37. Huckins C. Cell cycle properties of differentiating spermatogonia in adult Sprague-Dawley rats. Cell Tissue Kinet 1971; 4:139–154.
38. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F_1 hybrid mouse. Mutat Res 1993; 290:193–200.
39. Li H, MacLean G, Cameron D, Clagett-Dame M, Petkovich M. Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. PLOS ONE 2009; 4: e7501.
40. MacLean G, Li H, Metzger D, Chambon P, Petkovich M. Apoptotic extinction of germ cells in testes of *Cyp26b1* knockout mice. Endocrinology 2007; 148:4560–4567.
41. Trautmann E, Guerquin MJ, Duquenne C, Lahaye JB, Habert R, Livera G. Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. Cell Cycle 2008; 7:656–664.
42. Busada JT, Kaye EP, Renegar RH, Geyer CB. Retinoic acid induces multiple hallmarks of the prospermatogonia-to-spermatogonia transition in the neonatal mouse. Biol Reprod 2014; 90:64.
43. Endo T, Romer KA, Anderson EL, Baltus AE, de Rooij DG, Page DC. Periodic retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. Proc Natl Acad Sci U S A 2015; 112:E2347–E2356.
44. Niedenberger BA, Busada JT, Geyer CB. Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis. Reproduction 2015; 149:329–338.
45. Raverdeau M, Gely-Pernot A, Feret B, Dennefeld C, Benoit G, Davidson I, Chambon P, Mark M, Ghyselinck NB. Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. Proc Natl Acad Sci U S A 2012; 109:16582–16587.
46. van Pelt AM, de Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology 1991; 128:697–704.
47. Chiarini-Garcia H, Russell LD. High-resolution light microscopic characterization of mouse spermatogonia. Biol Reprod 2001; 65: 1170–1178.
48. Kluin PM, Kramer MF, de Rooij DG. Spermatogenesis in the immature mouse proceeds faster than in the adult. Int J Androl 1982; 5:282–294.
49. Sada A, Hasegawa K, Pin PH, Saga Y. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. Stem Cells 2012; 30: 280–291.
50. Sada A, Suzuki A, Suzuki H, Saga Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 2009; 325:1394–1398.
51. Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N, Griswold MD. Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. Biol Reprod 2015; 92:37.
52. Mithraprabhu S, Loveland KL. Control of KIT signalling in male germ cells: what can we learn from other systems? Reproduction 2009; 138: 743–757.
53. Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C, Griswold MD. 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 2008; 79:35–42.
54. Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 1996; 135:469–477.
55. Tedesco M, La Sala G, Barbagallo F, De Felici M, Farini D. STRA8 shuttles between nucleus and cytoplasm and displays transcriptional activity. J Biol Chem 2009; 284:35781–35793.
56. Ballow D, Meistrich ML, Matzuk M, Rajkovic A. *Sohlh1* is essential for spermatogonial differentiation. Dev Biol 2006; 294:161–167.
57. Ballow DJ, Xin Y, Choi Y, Pangas SA, Rajkovic A. *Sohlh2* is a germ cell-specific bHLH transcription factor. Gene Expr Patterns 2006; 6: 1014–1018.
58. Busada JT, Chappell VA, Niedenberger BA, Kaye EP, Keiper BD, Hogarth CA, Geyer CB. Retinoic acid regulates *Kit* translation during spermatogonial differentiation in the mouse. Dev Biol 2015; 397: 140–149.
59. Geyer CB, Eddy EM. Identification and characterization of *Rhox13*, a novel X-linked mouse homeobox gene. Gene 2008; 423:194–200.
60. Geyer CB, Saba R, Kato Y, Anderson AJ, Chappell VK, Saga Y, Eddy EM. *Rhox13* is translated in premeiotic germ cells in male and female mice and is regulated by NANOS2 in the male. Biol Reprod 2012; 86: 127.
61. Paniagua R, Nistal M. Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat 1984; 139(pt 3): 535–552.
62. Hermann BP, Sukhwani M, Hansel MC, Orwig KE. Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 2010; 139:479–493.
63. Chen LY, Brown PR, Willis WB, Eddy EM. Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 2014; 155:en2014-1406.
64. Fouchecourt S, Godet M, Sabido O, Durand P. Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. J Endocrinol 2006; 190:59–71.
65. Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, Vicini E. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction 2012; 143:325–332.
66. Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J 2011; 25:2604–2614.
67. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 2000; 287:1489–1493.
68. Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 2006; 74:314–321.
69. Oatley JM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 2007; 282:25842–25851.
70. Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, Salvatore G, Chiappetta G, Sferratore F, Fusco A, Santoro M. Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia. Int J Oncol 2000; 16:689–694.
71. Yang QE, Kim D, Kaucher A, Oatley MJ, Oatley JM. CXCL12–CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. J Cell Sci 2013; 126:1009–1020.
72. Barrios F, Filipponi D, Pellegrini M, Paronetto MP, Di Siena S, Geremia R, Rossi P, De Felici M, Jannini EA, Dolci S. Opposing effects of retinoic acid and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells. J Cell Sci 2010; 123:871–880.
73. Bowles J, Feng CW, Spiller C, Davidson TL, Jackson A, Koopman P. FGF9 suppresses meiosis and promotes male germ cell fate in mice. Dev Cell 2010; 19:440–449.
74. Hasegawa K, Saga Y. FGF8-FGFR1 signaling acts as a niche factor for maintaining undifferentiated spermatogonia in the mouse. Biol Reprod 2014; 91:145.
75. Ishii K, Kanatsu-Shinohara M, Toyokuni S, Shinohara T. FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of *Etv5* and *Bcl6b* through MAP2K1 activation. Development 2012; 139: 1734–1743.

76. Wu Z, Falciatori I, Molyneux LA, Richardson TE, Chapman KM, Hamra FK. Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. Biol Reprod 2009; 81:77–86.
77. Zhang Y, Wang S, Wang X, Liao S, Wu Y, Han C. Endogenously produced FGF2 is essential for the survival and proliferation of cultured mouse spermatogonial stem cells. Cell Res 2012; 22:773–776.
78. Wolbach SB, Howe PR. Tissue changes following deprivation of fat-soluble A vitamin. J Exp Med 1925; 42:753–777.
79. van Pelt AM, van Dissel-Emiliani FM, Gaemers IC, van der Burg MJ, Tanke HJ, de Rooij DG. Characteristics of A spermatogonia and preleptotene spermatocytes in the vitamin A-deficient rat testis. Biol Reprod 1995; 53:570–578.
80. Gaemers IC, Sonneveld E, van Pelt AM, Schrans BH, Themmen AP, van der Saag PT, de Rooij DG. The effect of 9-cis-retinoic acid on proliferation and differentiation of a spermatogonia and retinoid receptor gene expression in the vitamin A-deficient mouse testis. Endocrinology 1998; 139:4269–4276.
81. de Rooij DG. Proliferation and differentiation of spermatogonial stem cells. Reproduction 2001; 121:347–354.
82. Hogarth CA, Evanoff R, Snyder E, Kent T, Mitchell D, Small C, Amory JK, Griswold MD. Suppression of *Stra8* expression in the mouse gonad by WIN 18 446. Biol Reprod 2011; 84:957–965.
83. Hogarth CA, Evanoff R, Mitchell D, Kent T, Small C, Amory JK, Griswold MD. Turning a spermatogenic wave into a tsunami: synchronizing murine spermatogenesis using WIN 18 446. Biol Reprod 2013; 88:40.
84. Evans EB, Hogarth CA, Mitchell D, Griswold MD. Riding the spermatogenic wave: profiling gene expression within neonatal germ and Sertoli cells during a synchronized initial wave of spermatogenesis in mice. Biol Reprod 2014; 90:108.
85. Hogarth CA, Griswold MD. Retinoic acid regulation of male meiosis. Curr Opin Endocrinol Diabetes Obes 2013; 20:217–223.
86. Molotkov A, Ghyselinck NB, Chambon P, Duester G. Opposing actions of cellular retinol-binding protein and alcohol dehydrogenase control the balance between retinol storage and degradation. Biochem J 2004; 383: 295–302.
87. Theodosiou M, Laudet V, Schubert M. From carrot to clinic: an overview of the retinoic acid signaling pathway. Cell Mol Life Sci 2010; 67: 1423–1445.
88. Chung SS, Sung W, Wang X, Wolgemuth DJ. Retinoic acid receptor α is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. Dev Dyn 2004; 230:754–766.
89. Chung SS, Wang X, Wolgemuth DJ. Male sterility in mice lacking retinoic acid receptor α involves specific abnormalities in spermiogenesis. Differentiation 2005; 73:188–198.
90. Chung SS, Wang X, Wolgemuth DJ. Expression of retinoic acid receptor alpha in the germline is essential for proper cellular association and spermiogenesis during spermatogenesis. Development 2009; 136: 2091–2100.
91. de Bruijn DR, Oerlemans F, Hendriks W, Baats E, Ploemacher R, Wieringa B, Geurts van Kessel A. Normal development, growth and reproduction in cellular retinoic acid binding protein-I (CRABPI) null mutant mice. Differentiation 1994; 58:141–148.
92. Gely-Pernot A, Raverdeau M, Celebi C, Dennefeld C, Feret B, Klopfenstein M, Yoshida S, Ghyselinck NB, Mark M. Spermatogonia differentiation requires retinoic acid receptor gamma. Endocrinology 2012; 153:438–449.
93. Gorry P, Lufkin T, Dierich A, Rochette-Egly C, Decimo D, Dolle P, Mark M, Durand B, Chambon P. The cellular retinoic acid binding protein I is dispensable. Proc Natl Acad Sci U S A 1994; 91:9032–9036.
94. Hogarth CA, Evans E, Onken J, Kent T, Mitchell D, Petkovich M, Griswold MD. CYP26 enzymes are necessary within the postnatal seminiferous epithelium for normal murine spermatogenesis. Biol Reprod 2015; 93:19.
95. Lampron C, Rochette-Egly C, Gorry P, Dolle P, Mark M, Lufkin T, LeMeur M, Chambon P. Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development 1995; 121:539–548.
96. Luo J, Pasceri P, Conlon RA, Rossant J, Giguere V. Mice lacking all isoforms of retinoic acid receptor β develop normally and are susceptible to the teratogenic effects of retinoic acid. Mech Dev 1995; 53:61–71.
97. Snyder EM, Davis JC, Zhou Q, Evanoff R, Griswold MD. Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. Biol Reprod 2011; 84:886–893.
98. Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, Griswold MD. Expression of stimulated by retinoic acid gene 8 (*Stra8*) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. Biol Reprod 2008; 78:537–545.
99. Griswold MD, Hogarth CA, Bowles J, Koopman P. Initiating meiosis: the case for retinoic acid. Biol Reprod 2012; 86:35.
100. Huang HF, Marshall GR. Failure of spermatid release under various vitamin A states—an indication of delayed spermiation. Biol Reprod 1983; 28:1163–1172.
101. Tong MH, Yang QE, Davis JC, Griswold MD. Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. Proc Natl Acad Sci U S A 2013; 110:543–548.
102. Ikami K, Tokue M, Sugimoto R, Noda C, Kobayashi S, Hara K, Yoshida S. Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. Development 2015; 142:1582–1592.
103. Ghyselinck NB, Dupe V, Dierich A, Messaddeq N, Garnier JM, Rochette-Egly C, Chambon P, Mark M. Role of the retinoic acid receptor beta (RARbeta) during mouse development. Int J Dev Biol 1997; 41: 425–447.
104. Doyle TJ, Braun KW, McLean DJ, Wright RW, Griswold MD, Kim KH. Potential functions of retinoic acid receptor A in Sertoli cells and germ cells during spermatogenesis. Ann N Y Acad Sci 2007; 1120:114–130.
105. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, LeMeur M, Chambon P. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci U S A 1993; 90: 7225–7229.
106. Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM. Functional and molecular features of the $Id4^{+}$ germline stem cell population in mouse testes. Genes Dev 2014; 28:1351–1362.
107. Shima JE, McLean DJ, McCarrey JR, Griswold MD. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 2004; 71: 319–330.
108. Koubova J, Hu YC, Bhattacharyya T, Soh YQ, Gill ME, Goodheart ML, Hogarth CA, Griswold MD, Page DC. Retinoic acid activates two pathways required for meiosis in mice. PLOS Genet 2014; 10:e1004541.
109. Wang PJ, McCarrey JR, Yang F, Page DC. An abundance of X-linked genes expressed in spermatogonia. Nat Genet 2001; 27:422–426.
110. Wang PJ, Pan J. The role of spermatogonially expressed germ cell-specific genes in mammalian meiosis. Chromosome Res 2007; 15: 623–632.
111. Laronda MM, Jameson JL. Sox3 functions in a cell-autonomous manner to regulate spermatogonial differentiation in mice. Endocrinology 2011; 152:1606–1615.
112. Raverot G, Weiss J, Park SY, Hurley L, Jameson JL. Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 2005; 283:215–225.
113. Suzuki H, Ahn HW, Chu T, Bowden W, Gassei K, Orwig K, Rajkovic A. SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol 2012; 361:301–312.
114. Toyoda S, Miyazaki T, Miyazaki S, Yoshimura T, Yamamoto M, Tashiro F, Yamato E, Miyazaki J. *Sohlh2* affects differentiation of KIT positive oocytes and spermatogonia. Dev Biol 2009; 325:238–248.
115. Yoshida S. Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. Reproduction 2012; 144:293–302.
116. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004; 36:647–652.
117. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; 36:653–659.
118. Hermann BP, Mutoji KN, Velte EK, Ko D, Oatley JM, Geyer CB, McCarrey JR. Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. Biol Reprod 2015; 92:54.
119. Lodish HF. Translational control of protein synthesis. Annu Rev Biochem 1976; 45:39–72.
120. Busada JT, Niedenberger BA, Velte EK, Keiper BD, Geyer CB. Mammalian target of rapamycin complex 1 (mTORC1) is required for mouse spermatogonial differentiation in vivo. Dev Biol 2015; 407: 90–102.
121. Yang QE, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. Development 2013; 140:280–290.
122. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136:215–233.
123. Korhonen HM, Meikar O, Yadav RP, Papaioannou MD, Romero Y, Da

Ros M, Herrera PL, Toppari J, Nef S, Kotaja N. Dicer is required for haploid male germ cell differentiation in mice. PLOS ONE 2011; 6: e24821.
124. Liu D, Li L, Fu H, Li S, Li J. Inactivation of *Dicer1* has a severe cumulative impact on the formation of mature germ cells in mouse testes. Biochem Biophys Res Commun 2012; 422:114–120.
125. Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. *Dicer1* is required for differentiation of the mouse male germline. Biol Reprod 2008; 79:696–703.
126. Romero Y, Meikar O, Papaioannou MD, Conne B, Grey C, Weier M, Pralong F, De Massy B, Kaessmann H, Vassalli JD, Kotaja N, Nef S. *Dicer1* depletion in male germ cells leads to infertility due to cumulative meiotic and spermiogenic defects. PLOS ONE 2011; 6:e25241.
127. Wu Q, Song R, Ortogero N, Zheng H, Evanoff R, Small CL, Griswold MD, Namekawa SH, Royo H, Turner JM, Yan W. The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. J Biol Chem 2012; 287:25173–25190.
128. Huszar JM, Payne CJ. MicroRNA 146 (*Mir146*) modulates spermatogonial differentiation by retinoic acid in mice. Biol Reprod 2013; 88:15.
129. Tong MH, Mitchell D, Evanoff R, Griswold MD. Expression of *Mirlet7* family microRNAs in response to retinoic acid-induced spermatogonial differentiation in mice. Biol Reprod 2011; 85:189–197.
130. Tong MH, Mitchell DA, McGowan SD, Evanoff R, Griswold MD. Two miRNA clusters, *Mir-17-92* (*Mirc1*) and *Mir-106b-25* (*Mirc3*), are involved in the regulation of spermatogonial differentiation in mice. Biol Reprod 2012; 86:72.
131. Beck AR, Miller IJ, Anderson P. RNA-binding protein TIAR is essential for primordial germ cell development. Proc Natl Acad Sci U S A 1998; 95:2331–2336.
132. Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W, Lin H, Matsuda Y, et al. *Mili*, a mammalian member of *piwi* family gene, is essential for spermatogenesis. Development 2004; 131:839–849.
133. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Takamatsu K, Chuma S, Kojima-Kita K, Shiromoto Y, Asada N, Toyoda A, Fujiyama A, Totoki Y, Shibata T, et al. MVH in piRNA processing and gene silencing of retrotransposons. Genes Dev 2010; 24:887–892.
134. Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri TW, Hata K, Li E, et al. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev 2008; 22: 908–917.
135. Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ. The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. Nature 1997; 389:73–77.
136. Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y. Conserved role of nanos proteins in germ cell development. Science 2003; 301:1239–1241.
137. Suzuki A, Igarashi K, Aisaki K, Kanno J, Saga Y. NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs. Proc Natl Acad Sci U S A 2010; 107:3594–3599.
138. Zhou Z, Shirakawa T, Ohbo K, Sada A, Wu Q, Hasegawa K, Saba R, Saga Y. RNA Binding protein Nanos2 organizes post-transcriptional buffering system to retain primitive state of mouse spermatogonial stem cells. Dev Cell 2015; 34:96–107.
139. Suzuki A, Saga Y. Nanos2 suppresses meiosis and promotes male germ cell differentiation. Genes Dev 2008; 22:430–435.
140. Beamer WG, Cunliffe-Beamer TL, Shultz KL, Langley SH, Roderick TH. Juvenile spermatogonial depletion (jsd): a genetic defect of germ cell proliferation of male mice. Biol Reprod 1988; 38:899–908.
141. Bradley J, Baltus A, Skaletsky H, Royce-Tolland M, Dewar K, Page DC. An X-to-autosome retrogene is required for spermatogenesis in mice. Nat Genet 2004; 36:872–876.
142. Rohozinski J, Bishop CE. The mouse *juvenile spermatogonial depletion* (*jsd*) phenotype is due to a mutation in the X-derived retrogene, *mUtp14b*. Proc Natl Acad Sci U S A 2004; 101:11695–11700.
143. Bolden-Tiller OU, Chiarini-Garcia H, Poirier C, Alves-Freitas D, Weng CC, Shetty G, Meistrich ML. Genetic factors contributing to defective spermatogonial differentiation in juvenile spermatogonial depletion (*Utp14b*^{jsd}) mice. Biol Reprod 2007; 77:237–246.
144. Comish PB, Liang LY, Yamauchi Y, Weng CC, Shetty G, Naff KA, Ward MA, Meistrich ML. Increasing testicular temperature by exposure to elevated ambient temperatures restores spermatogenesis in adult *Utp14b*^{jsd} mutant (*jsd*) mice. Andrology 2015; 3:376–384.
145. Shetty G, Porter KL, Zhou W, Shao SH, Weng CC, Meistrich ML. Androgen suppression-induced stimulation of spermatogonial differentiation in juvenile spermatogonial depletion mice acts by elevating the testicular temperature. Endocrinology 2011; 152:3504–3514.
146. Shetty G, Weng CC. Cryptorchidism rescues spermatogonial differentiation in juvenile spermatogonial depletion (*jsd*) mice. Endocrinology 2004; 145:126–133.
147. Burgoyne PS, Levy ER, McLaren A. Spermatogenic failure in male mice lacking H-Y antigen. Nature 1986; 320:170–172.
148. Sutcliffe MJ, Burgoyne PS. Analysis of the testes of H-Y negative XOSxrb mice suggests that the spermatogenesis gene (Spy) acts during the differentiation of the A spermatogonia. Development 1989; 107: 373–380.
149. Matsubara Y, Kato T, Kashimada K, Tanaka H, Zhi Z, Ichinose S, Mizutani S, Morio T, Chiba T, Ito Y, Saga Y, Takada S, et al. TALEN-mediated gene disruption on Y chromosome reveals critical role of EIF2S3Y in mouse spermatogenesis. Stem Cells Dev 2015; 24: 1164–1170.
150. Mazeyrat S, Saut N, Grigoriev V, Mahadevaiah SK, Ojarikre OA, Rattigan A, Bishop C, Eicher EM, Mitchell MJ, Burgoyne PS. A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. Nat Genet 2001; 29:49–53.
151. Motomura K, Ohata M, Satre M, Tsukamoto H. Destabilization of TNF-alpha mRNA by retinoic acid in hepatic macrophages: implications for alcoholic liver disease. Am J Physiol Endocrinol Metab 2001; 281: E420–E429.
152. Yamauchi Y, Riel JM, Stoytcheva Z, Ward MA. Two Y genes can replace the entire Y chromosome for assisted reproduction in the mouse. Science 2014; 343:69–72.
153. Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. Global quantification of mammalian gene expression control. Nature 2011; 473:337–342.
154. Wagner A. Energy constraints on the evolution of gene expression. Mol Biol Evol 2005; 22:1365–1374.
155. Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. J Biol Chem 2002; 277:25297–25304.
156. Masia S, Alvarez S, de Lera AR, Barettino D. Rapid, nongenomic actions of retinoic acid on phosphatidylinositol-3-kinase signaling pathway mediated by the retinoic acid receptor. Mol Endocrinol 2007; 21: 2391–2402.
157. Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, Bachvarova RF. The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis.Dev Suppl 1993; 125–137.
158. Packer AI, Besmer P, Bachvarova RF. Kit ligand mediates survival of type A spermatogonia and dividing spermatocytes in postnatal mouse testes. Mol Reprod Dev 1995; 42:303–310.
159. Kissel H, Timokhina I, Hardy MP, Rothschild G, Tajima Y, Soares V, Angeles M, Whitlow SR, Manova K, Besmer P. Point mutation in Kit receptor tyrosine kinase reveals essential roles for Kit signaling in spermatogenesis and oogenesis without affecting other Kit responses. EMBO J 2000; 19:1312–1326.
160. Deshpande S, Agosti V, Manova K, Moore MA, Hardy MP, Besmer P. Kit ligand cytoplasmic domain is essential for basolateral sorting in vivo and has roles in spermatogenesis and hematopoiesis. Dev Biol 2010; 337: 199–210.
161. Hemmings BA, Restuccia DF. PI3K-PKB/Akt pathway. Cold Spring Harb Perspect Biol 2012; 4:a011189.
162. Clemens MJ. Targets and mechanisms for the regulation of translation in malignant transformation. Oncogene 2004; 23:3180–3188.
163. Rhoads RE. Signal transduction pathways that regulate eukaryotic protein synthesis. J Biol Chem 1999; 274:30337–30340.
164. Gallardo T, Shirley L, John GB, Castrillon DH. Generation of a germ cell-specific mouse transgenic Cre line, *Vasa-Cre*. Genesis 2007; 45: 413–417.
165. Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 2011; 121:3456–3466.
166. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 2003; 17: 1829–1834.