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Periodic retinoic acid–*STRA8* signaling intersects with periodic germ-cell competencies to regulate spermatogenesis

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Mammalian spermatogenesis—the transformation of stem cells into millions of haploid spermatozoa—is elaborately organized in time and space. We explored the underlying regulatory mechanisms by genetically and chemically perturbing spermatogenesis in vivo, focusing on spermatogonial differentiation, which begins a series of amplifying divisions, and meiotic initiation, which ends these divisions. We first found that, in mice lacking the retinoic acid (RA) target gene *Stimulated by retinoic acid gene 8 (Stra8)*, undifferentiated spermatogonia accumulated in unusually high numbers as early as 10 d after birth, whereas differentiating spermatogonia were depleted. We thus conclude that *Stra8*, previously shown to be required for meiotic initiation, also promotes (but is not strictly required for) spermatogonial differentiation. Second, we found that injection of RA into wild-type adult males induced, independently, precocious spermatogonial differentiation and precocious meiotic initiation; thus, RA acts instructively on germ cells at both transitions. Third, the competencies of germ cells to undergo spermatogonial differentiation or meiotic initiation in response to RA were found to be distinct, periodic, and limited to particular seminiferous stages. Competencies for both transitions begin while RA levels are low, so that the germ cells respond as soon as RA levels rise. Together with other findings, our results demonstrate that periodic RA–*STRA8* signaling intersects with periodic germ-cell competencies to regulate two distinct, cell-type-specific responses: spermatogonial differentiation and meiotic initiation. This simple mechanism, with one signal both starting and ending the amplifying divisions, contributes to the prodigious output of spermatozoa and to the elaborate organization of spermatogenesis.

spermatogenesis | *Stra8* | mouse | retinoic acid | testis

The adult mammalian testis is among the body's most proliferative tissues, producing millions of highly specialized gametes, or spermatozoa, each day. Spermatogenesis (the program of sperm production) is carefully regulated, ensuring that spermatozoa are produced at a constant rate. We used the mouse as a model to understand how mammalian spermatogenesis is organized at the cellular and molecular level. We focused on two key transitions: spermatogonial differentiation, which occurs cyclically and begins a series of programmed mitotic divisions, and meiotic initiation, which ends these divisions and marks the beginning of the meiotic program (Fig. 1A).

Like other proliferative tissues (e.g., blood, intestine, and skin), the testis relies on a modest number of stem cells (1, 2). The undifferentiated spermatogonia (also known as the A_{single} / A_{paired} / A_{aligned} spermatogonia), which encompass these stem cells, have a remarkable capacity for self-renewal and differentiation: They can reconstitute spermatogenesis upon transplantation to a germ-cell-depleted testis (3, 4). In vivo, undifferentiated spermatogonia ultimately give rise to a single cell type, spermatozoa, yet these undifferentiated spermatogonia express pluripotency-associated genes such as *Lin28a* (*Lin-28 homolog A*) (5) and *Pou5f1/Oct4* (6, 7) and are the only postnatal mammalian cells from which

functionally pluripotent cells have been derived in vitro without introduction of exogenous transcription factors or miRNAs (8). Undifferentiated spermatogonia periodically undergo spermatogonial differentiation (also known as the $A_{\text{aligned-to-}A_1}$ transition) to become differentiating spermatogonia (also known as $A_1/A_2/A_3/A_4$ /intermediate/B spermatogonia). During spermatogonial differentiation, the spermatogonia down-regulate pluripotency-associated genes (5, 9), lose capacity for self-renewal (4), and accelerate their cell cycle (10) to begin a series of six transit-amplifying mitotic divisions. At the conclusion of these mitotic divisions, germ cells become spermatocytes, and undergo meiotic initiation (Fig. 1A). This begins the meiotic program of DNA replication and reductive cell divisions, ensuring that spermatozoa contribute exactly one of each chromosome to the zygote.

Meiotic initiation is precisely coordinated with spermatogonial differentiation: The six mitotic divisions separating the two transitions occur over a span of exactly 8.6 d (11). Moreover, spermatogonial differentiation and meiotic initiation occur in close physical proximity. The testis comprises structures known as seminiferous tubules (Fig. S1A); while one generation of germ cells is initiating meiosis, a younger generation is simultaneously undergoing spermatogonial differentiation, within the same tubule

Significance

As male sex cells mature into sperm, two pivotal transitions are spermatogonial differentiation (exit from the stem cell pool) and meiotic initiation. These transitions occur in physical proximity, with 8.6-d periodicity. We report that the gene *Stra8*, essential for meiotic initiation, also promotes (but is not required for) spermatogonial differentiation. Moreover, injected RA induces both transitions to occur precociously. We conclude that a periodic RA signal, acting instructively through the common target *Stra8*, coordinates these transitions. This RA signal intersects with two distinct windows of sex-cell competency, which both begin while RA levels are low; sex cells respond quickly to rising RA. These mechanisms help account for the elaborate organization of sperm production, and its prodigious output.

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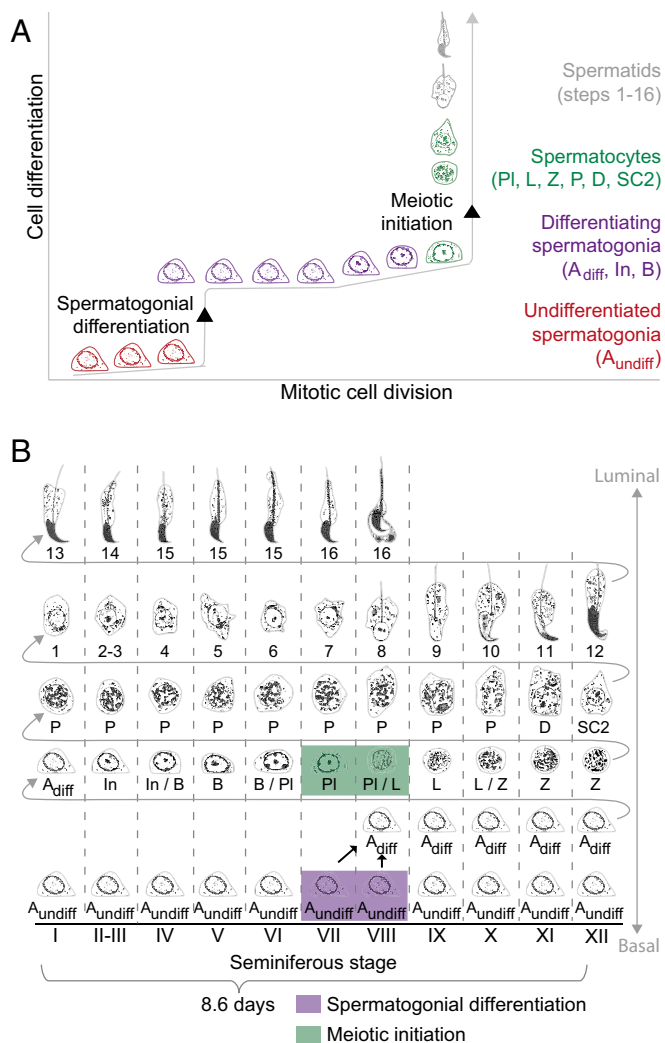


Fig. 1. Overview of spermatogenesis. (A) Diagram of mouse spermatogenesis. Germ cells develop from undifferentiated, mitotic spermatogonia (red, bottom left), to differentiating spermatogonia (purple, middle left), to spermatocytes (germ cells that undergo meiosis, green, middle right), to haploid spermatids (gray, top right). We highlight two cellular differentiation steps: spermatogonial differentiation and meiotic initiation. (B) Diagram of germ-cell associations (stages) in the mouse testis. Oakberg (33) identified 12 distinct cellular associations, called seminiferous stages I–XII. In the mouse, it takes 8.6 d for a section of the seminiferous tubule, and the germ cells contained within, to cycle through all 12 stages (41). Four turns of this seminiferous cycle are required for a germ cell to progress from undifferentiated spermatogonium to spermatozoon that is ready to be released into the tubule lumen. A_{undiff} , A_{diff} , In, and B: undifferentiated type A, differentiating type A, intermediate, and type B spermatogonia, respectively. D, L, P, Pl, SC2, and Z: diplotene, leptotene, pachytene, preleptotene, secondary spermatocytes, and zygotene, respectively. Steps 1–16: steps in spermatid differentiation. Purple: germ cells undergoing spermatogonial differentiation; green: meiotic initiation.

cross-section (Fig. 1B and Fig. S14). Spermatogonial differentiation and meiotic initiation occur in close association not only in mice but also in other mammals, including rats (12, 13), hamsters, and rams (14). This precise coordination of different steps of spermatogenesis is called the “cycle of the seminiferous epithelium” (or “seminiferous cycle”); it has fascinated biologists for over a century (15). We sought to explain the cooccurrence of spermatogonial differentiation and meiotic initiation, to better understand the regulation of these two transitions and the overall organization of the testis.

Both of these transitions require RA, a derivative of vitamin A. In vitamin A-deficient (VAD) mice and rats, most germ cells arrest as undifferentiated spermatogonia (16, 17). In VAD rat testes, some germ cells instead arrest just before meiosis, as preleptotene spermatocytes (16, 18). When VAD animals are injected with RA or vitamin A, the arrested spermatogonia differentiate (17–19), and the arrested preleptotene spermatocytes initiate meiosis (18). During spermatogonial differentiation, RA is believed to act, at least in part, directly on germ cells: Spermatogonia express RA receptors (RARs) (20), and genetic ablation of RARs in germ cells modestly impairs spermatogonial differentiation (21).

To understand how RA might coregulate these two transitions, we needed to understand its target genes. During meiotic initiation, RA acts instructively, through the target gene *Stra8* (Stimulated by retinoic acid gene 8). RA induces *Stra8* expression in germ cells—not in somatic cells—in both males and females (22, 23). *Stra8* is required for meiotic initiation in both sexes: *Stra8*-deficient germ cells in postnatal males and fetal females arrest just before meiosis, without entering meiotic prophase (24, 25). In contrast, no specific RA target genes have been implicated in spermatogonial differentiation: RA could either instruct the germ cells or simply be permissive for this transition. We considered *Stra8* as a candidate regulator of spermatogonial differentiation: STRA8 protein is expressed in spermatogonia as well as in preleptotene spermatocytes in vivo (26, 27), and in vitro studies suggest that RA can act directly on early spermatogonia to increase expression of *Stra8* (28). However, the functional role, if any, of *Stra8* in spermatogonia was not previously known. Using two complementary perturbations of RA–STRA8 signaling—genetic disruption of *Stra8* function and chemical manipulation of RA levels—we demonstrated that RA acts instructively, and at least in part through STRA8, at spermatogonial differentiation as well as at meiotic initiation. The shared RA–STRA8 signal helps to coordinate these two transitions in time and space.

Results

Massive Accumulations of Type A Spermatogonia in Testes of Aged *Stra8*-Deficient Males. As we previously reported, *Stra8*-deficient testes lacked meiotic and postmeiotic cells (24, 25); thus, at 8 wk of age, *Stra8*-deficient testes were much smaller than wild-type testes (25). However, we observed that, after 6 mo, some *Stra8*-deficient testes were grossly enlarged (>400 mg) (44%; 11 of 25 mice) compared with wild-type testes (91 ± 7 mg; average of testes from three mice) (Fig. 2A). Both small and large aged *Stra8*-deficient testes (88%; 22 of 25 mice) contained accumulated cells that resembled spermatogonia and expressed the germ-cell marker DAZL (Fig. 2B); these accumulations were absent in aged wild-type and heterozygous mice (0%, 0 of 10 mice). In wild-type testes, spermatogonia were confined to the basal lamina of seminiferous tubules, but even in small *Stra8*-deficient testes (<50 mg) occasional tubules were filled with presumptive spermatogonia, which sometimes spilled into the testicular interstitium. Large *Stra8*-deficient testes were composed almost entirely of presumptive spermatogonia, with few remnants of tubule structure. Spermatogonial morphology was very similar between small and large *Stra8*-deficient testes. We used mRNA sequencing (mRNA-Seq) to confirm that spermatogonia had accumulated in *Stra8*-deficient testes; known spermatogonial marker genes (29) were up-regulated in *Stra8*-deficient testes vs. wild-type testes (Fig. S24).

We classified more precisely the spermatogonia in these massive accumulations. Based on nuclear morphology, spermatogonia can be classified as type A, intermediate, or type B (Fig. 1A and B) (11). Type A includes the undifferentiated and early differentiating spermatogonia, whereas intermediate and type B encompass the later differentiating spermatogonia. The

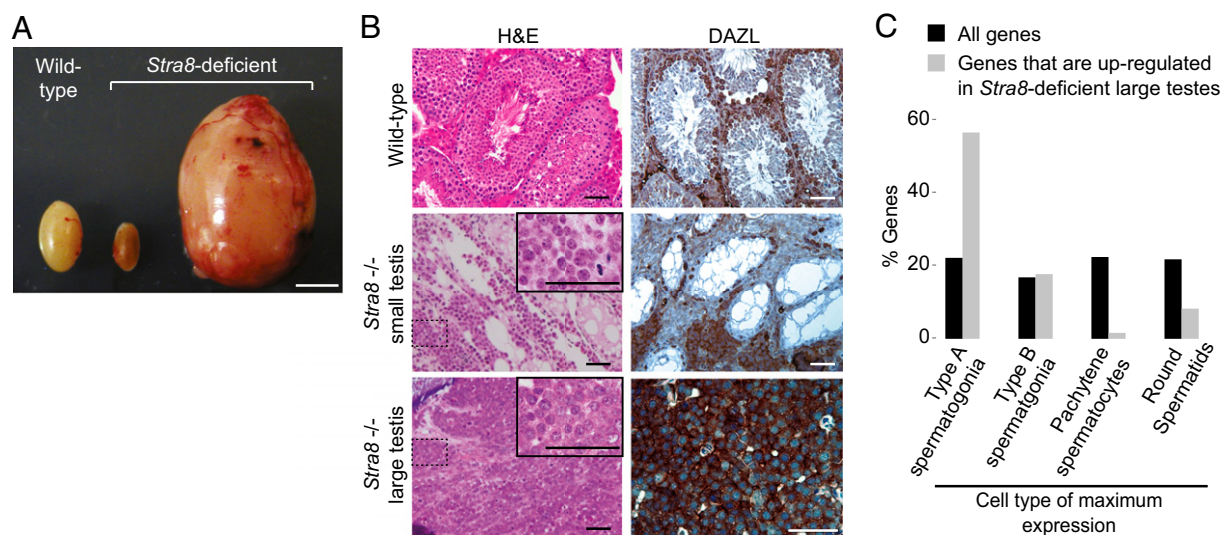


Fig. 2. Aged *Stra8*-deficient testes accumulate type A spermatogonia. (A) Wild-type (left) and *Stra8*-deficient small (center) and large (right) testes from 1-y-old mice. (Scale bar, 5 mm.) (B) Testis sections from 1-y-old mice: wild-type (Top), *Stra8*-deficient small testis (Middle), and *Stra8*-deficient grossly enlarged testis (Bottom). (Left) H&E staining (Right) DAZL immunostaining. Insets enlarge the boxed regions. (Scale bars, 50 μ m.) (C) Percentage of genes whose highest expression is found in type A spermatogonia, type B spermatogonia, pachytene spermatocytes, or round spermatids. Black bars (control), all analyzable genes (17,345 genes). Gray bars, 100 genes most significantly up-regulated in *Stra8*-deficient large testes relative to wild-type testes (Table S1).

Stra8-deficient spermatogonia had type A morphology (Fig. 2B). To confirm this, we used mRNA-Seq to identify the 100 genes most significantly up-regulated in *Stra8*-deficient vs. wild-type testes (Table S1). We analyzed their expression among different cell types in wild-type testes, using a published microarray dataset (30); 59% of these 100 genes were most highly expressed in type A spermatogonia, vs. 22.7% of a control gene set (all 17,345 genes on the microarray) (Fig. 2C) ($P < 0.001$, Fisher's exact test). A genome-wide clustering analysis of these data and other publically available datasets confirmed that the expression patterns of *Stra8*-deficient testes were overall quite similar to those of type A spermatogonia (Fig. S2B and SI Results and Discussion). We conclude that type A spermatogonia accumulate in *Stra8*-deficient testes. This suggests that STRA8 has a functional role in type A spermatogonia, distinct from its role in meiotic initiation.

Early Postnatal *Stra8*-Deficient Testes Contain Accumulations of Undifferentiated Spermatogonia and Are Depleted for Differentiating Spermatogonia. We considered our findings in light of published observations that (i) a subset of type A spermatogonia undergo spermatogonial differentiation, (ii) RA is required for spermatogonial differentiation (17, 18), and (iii) RA can act directly on spermatogonia to induce *Stra8* expression (28). We postulated that in the unperturbed wild-type testis, RA induction of *Stra8* promotes spermatogonial differentiation, whereas in the absence of *Stra8*, impaired spermatogonial differentiation leads to accumulation of undifferentiated spermatogonia, accounting for the massive accumulations of type A spermatogonia observed in aged *Stra8*-deficient males.

This hypothesis predicts that even in very young males *Stra8*-deficient testes should contain more undifferentiated spermatogonia than wild-type testes. To test this, we counted undifferentiated spermatogonia in testes from 10-d-old (p10) animals (Fig. 3A and B and Fig. S3A and B), using the markers LIN28A and PLZF (promyelocytic leukemia zinc finger, a.k.a. ZBTB16) (9, 31). As predicted, LIN28- and PLZF-positive spermatogonia were enriched in *Stra8*-deficient testes ($P < 10^{-15}$ for LIN28A, $P < 10^{-4}$ for PLZF, one-tailed Kolmogorov–Smirnov test) (Fig. 3B and Fig. S3B). *Stra8*-deficient testes had 9.4 ± 2.3 LIN28A-positive spermatogonia per tubule cross-section, vs. 4.4 ± 0.8 in wild-type testes ($P = 0.026$, one-tailed Welch's t test). As the testis matured, the

number of undifferentiated spermatogonia per tubule cross-section declined in both wild-type and *Stra8*-deficient testes, but undifferentiated spermatogonia remained significantly enriched in *Stra8*-deficient testes at p30 (Fig. 3C and Fig. S3C). Indeed, some testis tubules in p30 *Stra8*-deficient mice contained large clusters of LIN28A-positive and PLZF-positive type A spermatogonia (Fig. 3D and Fig. S3E). Spermatogonia in these clusters were densely packed in multiple layers, whereas in wild-type testes type A spermatogonia were widely spaced in a single layer (Fig. S3D). Thus, we conclude that undifferentiated spermatogonia progressively accumulate in *Stra8*-deficient animals. mRNA-Seq and immunohistochemical data from testes of aged *Stra8*-deficient mice were consistent with such an accumulation (Fig. S2B–E and SI Results and Discussion).

If this progressive accumulation were due to a defect in spermatogonial differentiation, the proportion of differentiating spermatogonia should be smaller in *Stra8*-deficient testes than in wild-type testes. We thus counted type B (differentiating) spermatogonia in *Stra8*-deficient and wild-type testes at p30 (Fig. 3C and Fig. S3F). Indeed, compared with wild-type testes, *Stra8*-deficient testes contained significantly fewer type B spermatogonia per tubule cross-section. As predicted, the ratio of differentiating-to-undifferentiated (LIN28-positive) spermatogonia was decreased to 2.8 in *Stra8*-deficient testes, vs. 7.9 in wild-type testes ($P = 0.05$ by Mann–Whitney U test). We conclude that STRA8 promotes (but is not strictly required for) spermatogonial differentiation.

STRA8 Expression Begins Shortly Before Spermatogonial Differentiation.

Previous reports showed that STRA8 is expressed in spermatogonia and spermatocytes but did not distinguish between different subtypes of spermatogonia (26, 27). We tested our model's prediction that STRA8 must be expressed before or during spermatogonial differentiation, immunostaining intact testis tubules for STRA8 and for PLZF (Fig. 4A and Fig. S4A). A subset of PLZF-positive (undifferentiated) spermatogonia expressed STRA8. We next immunostained for GFR α 1 (GDNF family receptor alpha 1), a marker of early undifferentiated spermatogonia (Fig. S4A) (32). GFR α 1 did not overlap with STRA8. We thus hypothesized that STRA8 expression begins immediately before spermatogonial differentiation.

To confirm this, we immunostained testis sections for STRA8 and then classified tubules by stage of the cycle of the seminif-

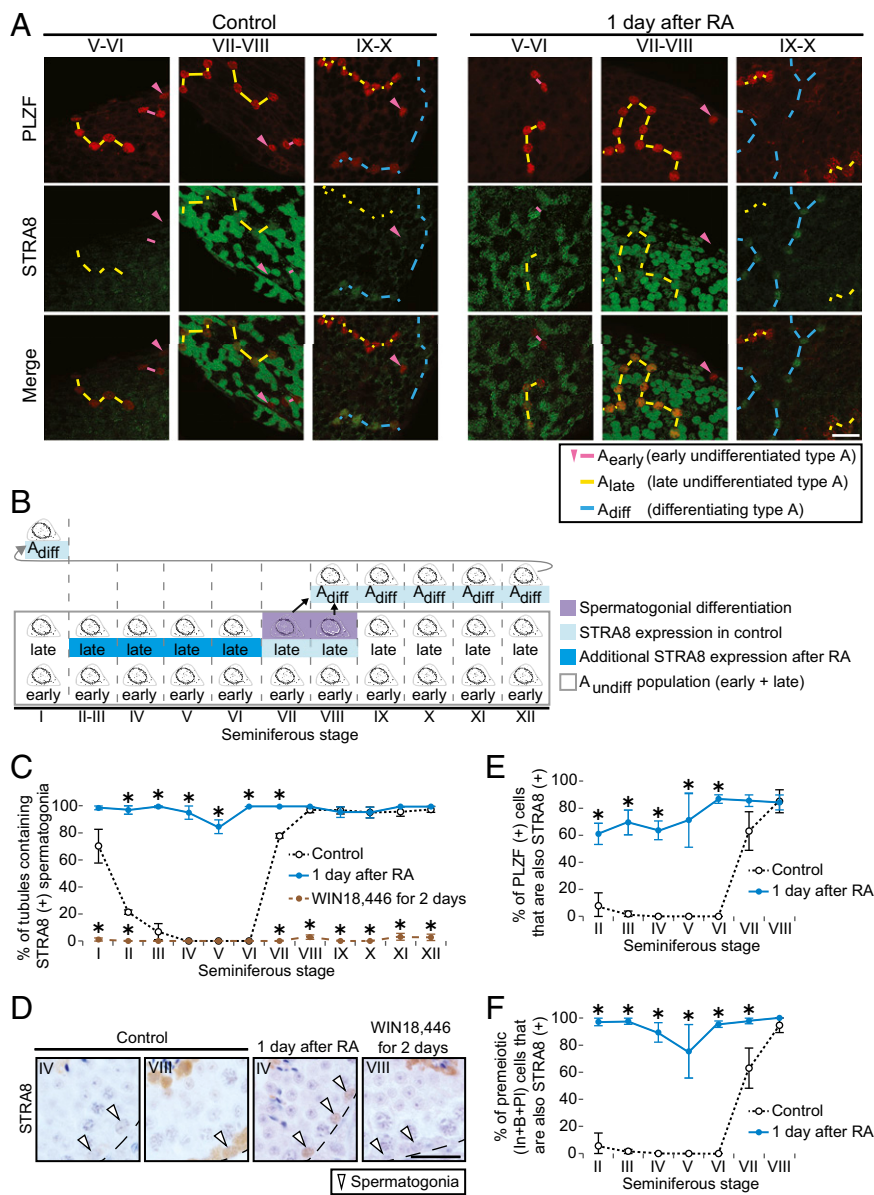


Fig. 4. STRA8 protein is normally present in late undifferentiated spermatogonia and can be precociously induced by injected RA. (A) Whole-mount immunostaining of intact wild-type testis tubules for PLZF (red) and STRA8 (green), in controls (Left) and 1 d after RA injection (Right). Arrowheads: isolated (single) spermatogonia. Dashed lines: putative interconnected chains of spermatogonia. Magenta labels: early undifferentiated type A spermatogonia (A_{early}). Yellow labels: late undifferentiated type A spermatogonia (A_{late}). Blue labels: differentiating type A spermatogonia (A_{diff}). (Scale bar, 30 μm .) (B) Diagram of STRA8 expression in type A spermatogonia, in controls (light blue) and 1 d after RA injection (light blue + dark blue). Diagram is based on observations in A and C. A_{undiff} , early, late, and A_{diff} : undifferentiated type A, early undifferentiated type A, late undifferentiated type A, and differentiating type A spermatogonia. (C) Percentage of testis tubule cross-sections containing STRA8-positive spermatogonia, in controls, 1 d after a single RA injection, and after 2 d of WIN18,446 treatment. Control data are duplicated from Fig. S1C. Error bars, mean \pm SD * P < 0.01 (one-tailed Welch's t test). (D) Immunostaining for STRA8 on testis cross-sections in stages IV and VIII. Dashed lines: basal laminae. Arrowheads: spermatogonia. (Scale bar, 30 μm .) (E and F) Percentage of PLZF-positive cells (E) or PLZF-negative premeiotic cells (F) that are also positive for STRA8 in testis cross-sections, in controls or 1 d after RA injection. Premeiotic germ cells (In+B+PI): intermediate and type B spermatogonia and preleptotene spermatocytes. Error bars, mean \pm SD * P < 0.01 (one-tailed Welch's t test).

Injected RA Induces Precocious Spermatogonial Differentiation. Because injected RA induced precocious STRA8 expression in stages II–VI, and STRA8 promotes spermatogonial differentiation, we hypothesized that RA would also induce spermatogonial differentiation in these stages. In the unperturbed testis, as a consequence of their differentiation in stages VII–VIII, spermatogonia express KIT protooncogene and enter mitotic S phase. They eventually develop into type B spermatogonia, then become preleptotene spermatocytes, and then initiate meiosis to become leptotene spermatocytes. We predicted that RA injection would cause undifferentiated spermatogonia to precociously begin this developmental progression.

We first confirmed that RA injection induced precocious KIT expression in spermatogonia. In control testis sections, KIT expression was absent in type A spermatogonia in stages II–VI and present in stages VII–VIII (Fig. S4E and G) (7). As predicted, at 1 d after RA injection, KIT was strongly induced in stages II–VI. We next tested for precocious entry into S phase, using PLZF to identify undifferentiated and newly differentiating spermatogonia, and BrdU incorporation to assay for S phase (Fig. S4F and H). Indeed, at 1 d after RA injection, many PLZF-positive spermatogonia

in stages II–VIII incorporated BrdU, whereas in control testes BrdU incorporation did not begin until stage VIII (10, 38).

If injected RA had induced precocious spermatogonial differentiation, the spermatogonia should develop into type B spermatogonia, preleptotene spermatocytes, and leptotene spermatocytes after 7, 8.6, and 10.6 d, respectively (Figs. 1B and 5A) (11). Thus, we should see transient increases in these cell types. As predicted, at 7 d after RA injection, type B spermatogonia were present in an increased fraction of testis tubules, in a much broader range of stages (XII–VI) than in control testes (IV–VI) (Fig. 5B and C and Fig. S5A). Preleptotene spermatocytes were similarly increased at 8.6 d after RA injection (in stages II–VIII, vs. VI–VIII in control testes) (Fig. 5D and E and Fig. S5B); throughout these stages, most of the premeiotic cells in the tubule cross-sections were preleptotene spermatocytes (Fig. S5D). Finally, at 10.6 d after RA injection, leptotene spermatocytes were present in an increased fraction of tubules, throughout stages VI–X (vs. VIII–X in control testes) (Fig. 5F and G and Fig. S5C). We confirmed our identification of leptotene spermatocytes throughout this broad range of stages by immunostaining for meiotic markers: γ H2AX (phosphorylated H2A histone family member X, a marker of DNA double strand breaks)

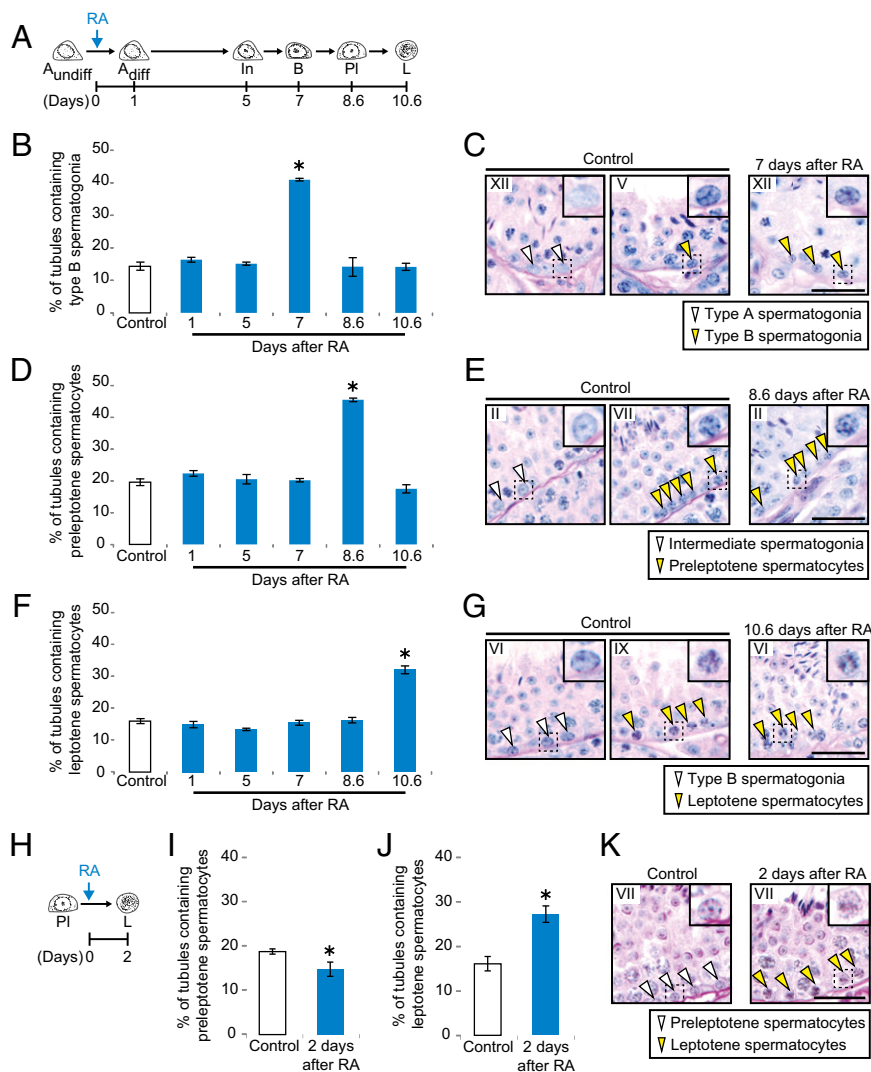


Fig. 5. Injected RA induces precocious spermatogonial differentiation and meiotic initiation. (A) Diagram of predicted germ-cell development after RA-induced spermatogonial differentiation. (B, D, and F) Percentage of tubules containing type B spermatogonia (B), preleptotene spermatocytes (D), or leptotene spermatocytes (F), in control or RA-injected testis cross-sections. Error bars, mean \pm SD * P < 0.01 compared with control (Dunnett's test). (C, E, and G) Control and RA-injected testis cross-sections, stained with hematoxylin and periodic acid-Schiff (He-PAS). Roman numerals indicate stages. Insets enlarge the boxed regions. Arrowheads in C: type A (white) and type B (yellow) spermatogonia. Arrowheads in E: intermediate spermatogonia (white) and preleptotene spermatocytes (yellow). Arrowheads in G: type B spermatogonia (white) and leptotene spermatocytes (yellow). (Scale bars, 30 μ m.) (H) Diagram of predicted germ-cell development after RA-induced meiotic initiation. (I and J) Percentage of tubules containing preleptotene (I) or leptotene (J) spermatocytes, in control or RA-injected testis cross-sections. Error bars, mean \pm SD * P < 0.01 compared with control (Dunnett's test). (K) Control and RA-injected testis cross-sections, stained with He-PAS. Roman numerals indicate stages. Insets enlarge boxed regions. Arrowheads: preleptotene (white) and leptotene (yellow) spermatocytes. (Scale bars, 30 μ m.)

(39) and SYCP3 (synaptonemal complex protein 3) (40). Indeed, at 10.6 d after RA injection, leptotene spermatocytes in stages VI–X were γ H2AX- and SYCP3-positive (Fig. 6A). The stages at which type B spermatogonia, preleptotene spermatocytes, and leptotene spermatocytes appeared after RA injection were completely consistent with spermatogonial differentiation having occurred throughout stages II–VIII (Fig. S5 E–G and Table S2).

We conclude that injected RA induced precocious spermatogonial differentiation. The precociously differentiated spermatogonia then progressed into meiotic prophase, ahead of schedule. Spermatogonial differentiation was limited to stages II–VIII, whereas undifferentiated spermatogonia in stages IX–I were seemingly unaffected by RA.

Injected RA Induces Precocious Meiotic Initiation. Because injected RA induces precocious STRA8 expression in both premeiotic cells and undifferentiated spermatogonia, and STRA8 is required for meiotic initiation, we hypothesized that injected RA would also induce precocious meiotic initiation. In the unperturbed testis, germ cells initiate meiosis in late stage VII and stage VIII and then develop into leptotene spermatocytes 2 d later. Thus, we expect a transient increase in leptotene spermatocytes at 2 d after RA injection. Indeed, leptotene spermatocytes were present in an increased fraction of testis tubules, in a broader range of stages (VII–X) than in control testes (VIII–X). The

percentage of tubules containing preleptotene spermatocytes was correspondingly decreased (Fig. 5 H–K and Fig. S5H). The precociously leptotene cells had normal meiotic γ H2AX and SYCP3 expression patterns (Fig. 6B). To confirm that precocious meiotic initiation was a specific effect of RA–STRA8 signaling, we used WIN18,446 to chemically block RA synthesis and inhibit STRA8 expression (Fig. S5I). As expected, WIN18,446 prevented meiotic initiation in preleptotene spermatocytes (Fig. S5J and K). We also confirmed that the precocious leptotene spermatocytes could progress normally through meiosis (Fig. S6 and SI Results and Discussion).

Our results show that premeiotic cells initiated meiosis precociously in response to injected RA and then progressed normally through meiosis, ahead of their usual schedule. However, precocious meiotic initiation occurred in fewer tubules than precocious spermatogonial differentiation (Fig. 5 B and J), strongly suggesting that the window of competence for meiotic initiation was narrower than that for spermatogonial differentiation. Based on the stages in which leptotene spermatocytes, zygotene spermatocytes, and meiotically dividing cells appeared after RA injection, we calculate that precocious meiotic initiation occurred in stage VI, and perhaps also in stages IV–V. This contrasts with precocious spermatogonial differentiation, which occurred throughout stages II–VI. Moreover, only premeiotic cells, not undifferentiated spermatogonia, were able to initiate

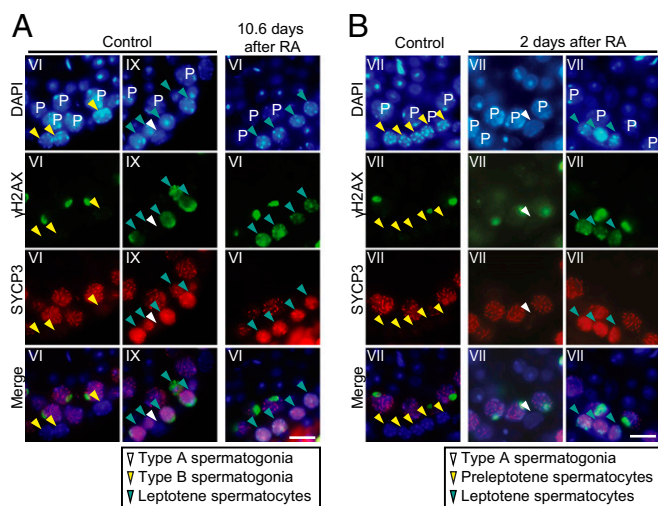


Fig. 6. Injected RA induces precocious expression of meiotic markers. (A and B) Immunostaining for γ H2AX (green) and SYCP3 (red), with DAPI counterstain (blue), on control and RA-injected testis cross-sections. Roman numerals indicate stages. Arrowheads in A: type A spermatogonia (white), type B spermatogonia (yellow), and leptotene spermatocytes (green). Arrowheads in B: type A spermatogonia (white), preleptotene spermatocytes (yellow), and leptotene spermatocytes (green). P: pachytene spermatocytes. (Scale bars, 10 μ m.)

meiosis directly in response to RA, as judged by the absence of γ H2AX and SYCP3 signals in type A spermatogonia, 2 d after RA injection (Fig. 6B). Thus, the competencies of germ cells to interpret the RA–STRA8 signal are distinct between undifferentiated spermatogonia and premeiotic cells.

Competencies for Spermatogonial Differentiation and Meiotic Initiation Are Limited to Distinct Subsets of Germ Cells. We set out to verify these distinct competencies using more stringent criteria. In the unperturbed testis, the seminiferous cycle lasts 8.6 d (i.e., in a given tubule section, spermatogonial differentiation and meiotic initiation occur once every 8.6 d) (Fig. 1B). We administered successive RA injections, once per 8.6-d cycle (Fig. 7A). We then predicted when different germ-cell types should appear, based on our findings that competence for spermatogonial differentiation was limited to stages II–VIII, that competence for meiotic initiation was limited to a subset of these stages, and that germ cells developed normally after precocious spermatogonial differentiation/meiotic initiation.

Competence for meiotic initiation. We first predicted that premeiotic cells in stages VI–VIII (and possibly also in stages IV/V), having initiated meiosis, would develop into step 7–8 spermatids after two 8.6-d intervals of RA injection (2×8.6 d) (Fig. 1B). Indeed, an increased percentage of tubules contained step 7–8 spermatids; step 6 spermatids were correspondingly depleted (Fig. S7A). Step 2–5 spermatids were virtually unchanged, demonstrating that meiotic initiation occurred specifically in stages VI–VIII, not in stages IV–V.

Competence for spermatogonial differentiation. We next predicted that spermatogonia in stages II–VIII, having differentiated, would develop into step 7–8 spermatids after 3×8.6 d of RA injection, then develop into spermatozoa after 4×8.6 d, and finally be released into the tubule lumen (Fig. 1B). Indeed, we saw increases in step 7–8 spermatids and spermatozoa after 3×8.6 d and 4×8.6 d, respectively (Fig. 7B and C and Fig. S7A). Successive RA injections were able to repeatedly induce spermatogonial differentiation; at 4×8.6 d, spermatozoa combined with younger RA-induced generations of germ cells to produce an excess of stage VII/VIII germ-cell associations, with a corresponding depletion of stages II–VI (Fig. 7B). Finally, after

4×8.6 plus 2 d of RA injection (36.4 d total), an increased percentage of tubules had released their spermatozoa (Fig. S7B–D). All these results are entirely consistent with competence for spermatogonial differentiation being limited to stages II–VIII.

Competence for neither. Finally, because germ cells in stages IX–I are competent for neither spermatogonial differentiation nor meiotic initiation, they should be unaffected by successive RA

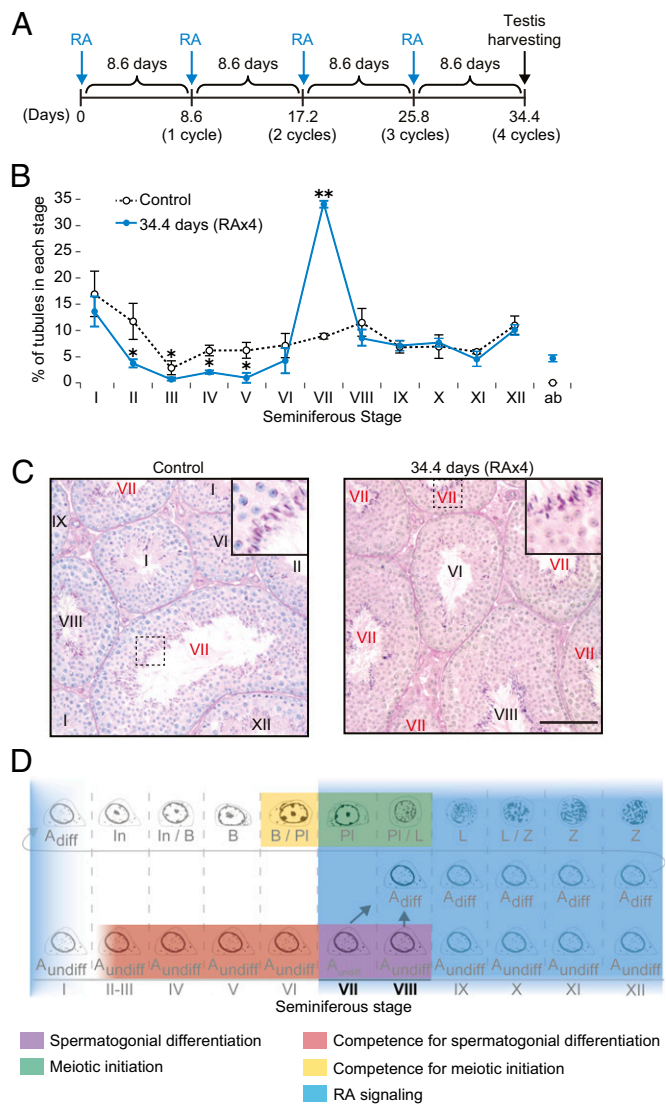


Fig. 7. Periodic RA–STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. (A) Diagram of four successive RA injections. Testes were harvested 34.4 d after the first RA injection. (B) Percentage of tubules in each stage of seminiferous cycle, in controls and after four RA injections. Abnormal germ-cell associations indicated by “ab”; all other germ-cell associations are fully normal. Error bars, mean \pm SD * P < 0.05, ** P < 0.01 (two-tailed Welch’s t test). (C) Testis cross-sections, stained with He-PAS, in controls (Left) or after four RA injections (Right). Cross-sections with the highest frequency of stage VII tubules (red) were selected. Stage VII tubules contain spermatozoa, clustered around the tubule lumen. Roman numerals indicate stages. Insets enlarge boxed regions. (Scale bar, 100 μ m.) (D) Model: Periodic RA–STRA8 signaling and periodic germ-cell competencies regulate spermatogonial differentiation and meiotic initiation. Red: competence for spermatogonial differentiation. Yellow: competence for meiotic initiation. Blue: stages in which we infer RA signaling is active. Competence for spermatogonial differentiation intersects with RA signaling to induce spermatogonial differentiation (purple). Competence for meiotic initiation intersects with RA signaling to induce meiotic initiation (green).

injections. Indeed, after 4×8.6 d of RA injections, the frequency of stage IX–I tubules was the same as in controls (Fig. 7B).

We found that, when germ cells are provided with RA, competence to undergo spermatogonial differentiation is strictly limited to stages II–VIII, whereas competence to undergo meiotic initiation is strictly limited to stages VI–VIII. The accuracy of our predictions, over long time scales, demonstrated that these windows of competence are precise. Furthermore, germ cells were able to develop at their normal pace after precocious spermatogonial differentiation/meiotic initiation. This development occurred even when germ cells were outside of their usual cell associations. Injected RA is thus able to accelerate spermatogenesis. Finally, we note that successive RA injections, combined with intrinsic germ-cell competencies, repeatedly induced spermatogonial differentiation; four successive injections were thus able to reestablish normal stage VII/VIII germ-cell associations (Fig. 7B and C). We conclude that spermatogonial differentiation and meiotic initiation are regulated by a shared RA–STRA8 signal intersecting with two distinct germ-cell competencies (Fig. 7D).

Discussion

RA–STRA8 Signaling Coordinates Spermatogonial Differentiation and Meiotic Initiation. Spermatogenesis in rodents is elaborately organized, with multiple generations of germ cells developing in stereotypical cell associations. This organization was first reported in 1888 (15) and by the 1950s had been comprehensively described (13, 33, 41). To understand spermatogenesis, we must systematically perturb its organization. Here, we used two complementary perturbations, genetic ablation of *Stra8* function and chemical manipulation of RA levels, to probe the coordination of two key transitions: spermatogonial differentiation and meiotic initiation.

We report that RA–STRA8 signaling plays an instructive role in both spermatogonial differentiation and meiotic initiation, inducing these transitions to occur together. Specifically, we provide the first functional evidence to our knowledge that STRA8, an RA target gene, promotes spermatogonial differentiation (as well as being required for meiotic initiation) (24, 25). In the absence of *Stra8*, spermatogonial differentiation was impaired: Undifferentiated spermatogonia began to accumulate as early as p10, ultimately giving rise to massive accumulations of type A spermatogonia in aged testes. These findings show that RA acts instructively at spermatogonial differentiation, by altering gene expression in spermatogonia. Genetic ablation of *Stra8* did not completely block spermatogonial differentiation, indicating that RA must act through additional targets at this transition. Additional targets could be activated either directly by RARs in spermatogonia, or indirectly, by the action of RA on the supporting somatic (Sertoli) cells of testis. Indeed, indirect RA signaling, via RAR α in Sertoli cells, is critical for the first round of spermatogonial differentiation (42). We also report that, in wild-type mice, RA injection induced precocious spermatogonial differentiation and meiotic initiation. We infer that, in the unperturbed wild-type testis, a single pulse of RA signaling drives STRA8 expression in both undifferentiated spermatogonia and premeiotic spermatocytes and induces two distinct, cell-type-specific responses. This shared RA–STRA8 signal helps to ensure that spermatogonial differentiation and meiotic initiation occur at the same time and place (Fig. 7D).

Evidence of Elevated RA Concentration in Stages VII–XII/I. In any given tubule cross-section, spermatogonial differentiation and meiotic initiation occur periodically, once every 8.6 d. Sugimoto et al. (43) and Hogarth et al. (44) have hypothesized that RA concentration also varies periodically over the course of this 8.6-d cycle. This hypothesis is supported by expression data, functional studies, and direct measurements of RA levels (34, 43, 45, 46). However, the pattern of RA periodicity was previously unclear. Hogarth et al. (34, 44) suggested a sharp RA peak in

stages VIII–IX. In contrast, based on expression patterns of RA-responsive genes and the functional consequences of inhibiting RA signaling, Hasegawa and Saga (45) suggested that RA levels rise in stage VII and remain high through stage XII.

Our data support the latter model, of a prolonged elevation of RA levels (Fig. 7D). We and others (35) have demonstrated that, in the unperturbed testis, STRA8 is periodically expressed and is present for the majority of the seminiferous cycle. Specifically, we show that STRA8 protein is present in spermatogonia in stages VII–XII/I and absent in II–VI. Furthermore, we show that, at the level of the tubule cross-section, spermatogonial STRA8 expression marks the presence of RA: When we increased RA levels by injecting RA or decreased them by injecting WIN18,446, STRA8 expression was immediately induced or repressed in all seminiferous stages (Fig. 4C). We thus agree with and extend the model of Hasegawa and Saga (45): In the unperturbed testis, RA levels rise in stage VII, rapidly inducing STRA8 and then inducing spermatogonial differentiation and meiotic initiation. RA levels remain high until stages XII/I. This model of a long RA–STRA8 pulse is consistent with additional published data. First, the enzyme *Aldh1a2*, which increases RA levels, is strongly expressed in stages VII–XII, whereas the enzymes *Lrat* and *Adfp*, which reduce RA levels, are expressed in stages I–VI/VII (43, 46). Second, although measured RA levels seem to peak in stages VIII–IX, they remain elevated for an extended period (2–4 d in pubertal animals, and through stage XII in adults) (34). Despite these persistently elevated RA levels, neither spermatogonial differentiation nor meiotic initiation recurs in stages IX–I. As we will now discuss, germ cells at these later stages lack competence for these transitions.

Undifferentiated Spermatogonia and Premeiotic Cells Have Different Competencies to Respond to RA–STRA8 Signaling. By examining responses to exogenous RA, we provide functional evidence that germ cells have periodic, stage-limited competencies to undergo spermatogonial differentiation and meiotic initiation. These competencies intersect with instructive, periodic RA–STRA8 signaling. Specifically, undifferentiated spermatogonia are competent for spermatogonial differentiation in stages II–VIII, and premeiotic cells are competent for meiotic initiation in stages VI–VIII (Fig. 7D). Competencies for both transitions begin while RA levels are low, so that the germ cells respond as soon as RA levels rise. Competencies for both transitions end simultaneously, while RA levels are still high. Thus, germ-cell competencies and high RA levels intersect briefly, causing spermatogonial differentiation and meiotic initiation to occur at the same time and place (in stages VII–VIII).

We also conclude that undifferentiated spermatogonia and premeiotic cells enact different molecular and cellular programs in response to RA–STRA8 signaling. In response to injected RA, only preleptotene spermatocytes (and possibly late type B spermatogonia, the immediate precursors of preleptotene spermatocytes) began to express meiotic markers such as SYCP3 and γ H2AX. Undifferentiated spermatogonia were not competent to initiate meiosis directly. Instead, in response to injected RA, most late undifferentiated spermatogonia began a program of spermatogonial differentiation, followed by six mitotic cell divisions. The early undifferentiated spermatogonia and a fraction of the late undifferentiated spermatogonia were seemingly unaffected by RA; they did not express STRA8 and did not differentiate. We believe that these undifferentiated spermatogonia are able to self-renew and proliferate even in the presence of RA, preventing the pool of undifferentiated spermatogonia from becoming depleted. Indeed, a normal complement of germ cells remained after repeated RA injections, indicating that injected RA did not eliminate the pool of undifferentiated spermatogonia (which includes the spermatogonial stem cells). Thus, distinct germ-cell competencies enable a single RA signal to induce both

spermatogonial differentiation and meiotic initiation and ensure that a subset of spermatogonia are able to self-renew and proliferate despite exposure to the RA signal.

We do not yet know the molecular mechanism behind the stage- and cell-type-specific competencies to differentiate in response to RA. These competencies cannot simply be explained by RAR expression, because the RARs do not have precise stage-specific expression patterns. For instance, RAR γ expression can be observed in all stages of the seminiferous cycle (21, 46). The competencies must therefore result from other aspects of germ-cell state. We note that competence for spermatogonial differentiation is closely correlated with proliferative activity. Specifically, undifferentiated spermatogonia in stages II–VIII, which are competent for differentiation, are arrested in the G0/G1 phase of the cell cycle, whereas undifferentiated spermatogonia in stages IX–I are actively proliferating (10, 38). Further studies are needed to identify the mechanisms by which competencies to undergo spermatogonial differentiation, and then meiotic initiation, are achieved.

The critical role for intrinsic germ-cell competence during spermatogenesis is in some respects analogous to its role of competence during oogenesis. In adult ovaries, immature oocytes are arrested at the diplotene stage of meiotic prophase. Some of these arrested oocytes grow and acquire intrinsic competence to resume meiosis and then acquire competence to mature (i.e., to progress to metaphase II arrest) (47, 48). These serially acquired competencies intersect with extrinsic, hormonal signals. We suggest that, in both oogenesis and spermatogenesis, properly timed differentiation depends on the intersection of extrinsic chemical cues and intrinsic competence.

RA–STRA8 Signaling Can Both Perturb and Reestablish the Complex Organization of the Testis. We find that injected RA can induce spermatogonial differentiation and meiotic initiation to occur precociously and ectopically, outside of their normal context. In the unperturbed testis, these two transitions occur together at stages VII/VIII, but, following a single RA injection, they occurred in different stages, with spermatogonial differentiation as early as stage II and meiotic initiation as early as stage VI. Then, when provided with RA at 8.6-d intervals, these precociously advancing germ cells were able to complete meiosis and develop into spermatozoa, ahead of schedule. This developmental flexibility is surprising, given the seemingly rigid organization of spermatogenesis (11). In the unperturbed testis, multiple generations of germ cells occur together in stereotypical associations; these associations are conserved across mammals and, before this study, had proven difficult to chemically disrupt (43, 49, 50). Nevertheless, when provided with RA, germ cells proceeded through spermatogenesis, outside of their usual environs, with no apparent guidance from the neighboring germ cells.

Why is spermatogenesis so precisely organized, if the stereotypical associations are not required for germ-cell development? We posit that this precise organization is in part a by-product of RA–STRA8 signaling (and germ-cell competencies): Cooccurrence of spermatogonial differentiation and meiotic initiation nucleates the stereotypical germ-cell associations. In support of this idea, when we administered successive RA injections at 8.6-d intervals, to repeatedly drive precocious spermatogonial differentiation and meiotic initiation, we were able to perturb and reestablish the characteristic germ-cell associations *in vivo*. The stereotypical associations, established by RA–STRA8 signaling, may ensure the efficiency of spermatogenesis.

We conclude that a simple regulatory mechanism helps to explain the testis's extraordinary capacity for proliferation and differentiation. Periodic RA signaling repeatedly induces spermatogonial differentiation and meiotic initiation, driving germ cells toward becoming highly specialized haploid spermatozoa. Meanwhile, distinct germ-cell competencies enforce that every

spermatogonium undergoes programmed amplifying divisions before initiating meiosis, guaranteeing a prodigious output of spermatozoa. Moreover, a fraction of spermatogonia undergo neither spermatogonial differentiation nor meiotic initiation in response to RA, ensuring that a reservoir of undifferentiated spermatogonia is maintained throughout the animal's reproductive lifetime. This basic understanding of the organization of spermatogenesis, derived from genetic and chemical perturbations, will facilitate future studies of germ-cell development, RA-driven differentiation, and cell competence, both *in vivo* and *in vitro*.

Materials and Methods

Mice. Three types of mice were used: wild-type (C57BL/6NtacFBR), *Strab8*-deficient (extensively back-crossed to C57BL/6) (26, 27), and *Dmc1*-deficient (B6.Cg-Dmc1^{tm1Jcs/JcsJ}) (51). See *SI Materials and Methods* for strain and genotyping details. Unless otherwise noted, experiments were performed on 6- to 8-wk-old male mice, fed a regular (vitamin A-sufficient) diet. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Statistics. Data are represented as mean \pm SD of three biological replicates. To compare two groups, Welch's *t* test (one- or two-tailed as indicated) or the Mann–Whitney *U* test were used. To compare three or more groups, one-way ANOVA with the Tukey–Kramer post hoc test was used. To compare multiple experimental groups with a control group, one-way ANOVA with Dunnett's post hoc test was used. To compare distributions, the Kolmogorov–Smirnov test was used. When performing genome-wide analysis of mRNA-Seq data, the Benjamini–Hochberg procedure was used to control the false discovery rate.

mRNA-Seq Sample Preparation. Testes were stripped of the tunica albuginea, placed in TRIzol (Invitrogen), homogenized, and stored at -20°C . Total RNAs were prepared according to the manufacturer's protocol. Total RNAs were then DNase-treated using DNA Free Turbo (Ambion). Libraries were prepared using the Illumina mRNA-Seq Sample Preparation Kit according to the manufacturer's protocol. Libraries were validated with an Agilent Bioanalyzer. Libraries were diluted to 10 pM and applied to an Illumina flow cell using the Illumina Cluster Station. The Illumina Genome Analyzer II platform was used to sequence 36-mers (single end) from the mRNA-Seq libraries.

mRNA-Seq and Microarray Data Analysis. For mRNA-Seq data, reads were aligned to the mouse genome using TopHat (52). Analysis was performed using edgeR (53), Cufflinks (54), and custom R scripts. Microarray data were normalized with the GCRMA package from Bioconductor, and replicates were averaged using limma (55). Comparison mRNA-Seq and microarray datasets were downloaded from National Center for Biotechnology Information GEO and Sequence Read Archive (SRA). See *SI Materials and Methods* for details on mRNA-Seq and microarray data processing and comparison.

Histology. Testes were fixed overnight in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, or with hematoxylin and periodic acid-Schiff (PAS). All sections were examined using a light microscope. Germ-cell types were identified by their location, nuclear size, and chromatin pattern (11). See *SI Materials and Methods* for details on identification of the stages of the seminiferous cycle.

Chemical Treatments. For RA injection experiments, mice received *i.p.* injections of 100 μL of 7.5 mg/mL all-*trans* RA (Sigma-Aldrich) in 16% (vol/vol) DMSO–H₂O. For BrdU incorporation experiments, mice received *i.p.* injections of 10 $\mu\text{L/g}$ body weight of 10 mg/mL BrdU (Sigma-Aldrich) in PBS, 4 h before they were killed. For WIN18,446 injection experiments, mice received *i.p.* injections of 100 μL of 20 mg/mL WIN18,446 (sc-295819A; Santa Cruz Biotechnology) in 16% DMSO–H₂O; mice were dosed at intervals of 12 h for a total of 2 or 4 d.

Immunostaining on Testis Sections. Testes were fixed overnight in Bouin's solution or 4% (wt/vol) paraformaldehyde, embedded in paraffin, and sectioned at 5- μm thickness. Slides were dewaxed, rehydrated, and heated in 10 mM sodium citrate buffer (pH 6.0). Sections were then blocked, incubated with the primary antibody, washed with PBS, incubated with the secondary antibody, and washed with PBS. Detection was fluorescent or

colorimetric. Antibodies and incubation conditions are provided in *SI Materials and Methods* and in *Table S3*.

Immunostaining on Intact Testis Tubules. Testes were stripped of the tunica albuginea, dispersed in PBS, fixed overnight in 4% paraformaldehyde at 4 °C, and washed with PBS. Testes were blocked with 2.5% (vol/vol) donkey serum, incubated with the primary antibody, washed with PBS, incubated with the secondary antibody, and washed with PBS. Finally, seminiferous tubules

were dissected from testes and mounted with SlowFade Gold antifade reagent with DAPI (S36939; Life Technologies). Antibodies and incubation conditions are provided in *SI Materials and Methods*.

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