Periodic production of retinoic acid by meiotic and somatic cells coordinates four transitions in mouse spermatogenesis

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Mammalian spermatogenesis is an elaborately organized differenti- ation process, starting with diploid spermatogonia, which include germ-line stem cells, and ending with haploid spermatozoa. The process involves four pivotal transitions occurring in physical prox- imity: spermatogonial differentiation, meiotic initiation, initiation of sperma- tid elongation, and release of spermatozoa. We report how the four transitions are coordinated in mice. Two premeiotic transi- tions, spermatogonial differentiation and meiotic initiation, were known to be coregulated by an extrinsic signal, retinoic acid (RA). Our chemical manipulations of RA levels in mouse testes now reveal that RA also regulates the two postmeiotic transitions: initiation of spermatid elongation and spermatozoa release. We measured RA concentrations and found that they changed periodically, as also reflected in the expression patterns of an RA-responsive gene, STRA8; RA levels were low before the four transitions, increased when the transitions occurred, and remained elevated thereafter. We found that pachytene spermatocytes, which express an RA-synthesizing enzyme, Aldh1a2, contribute directly and significantly to RA produc- tion in testes. Indeed, chemical and genetic depletion of pachytene spermatocytes revealed that RA from pachytene spermatocytes was required for the two postmeiotic transitions, but not for the two premeiotic transitions. We conclude that the premeiotic transitions are coordinated by RA from Sertoli (somatic) cells. Once germ cells enter meiosis, pachytene spermatocytes produce RA to coordinate the two postmeiotic transitions. In combination, these elements under- pin the spatiotemporal coordination of spermatogenesis and ensure its prodigious output in adult males.

retinoic acid | spermatogenesis | mouse | testis

Mammalian spermatogenesis is a choreographed process in which diploid spermatogonia undergo differentiation to give rise to specialized haploid gametes, called spermatozoa. Within the testis, several key developmental transitions of spermatogenesis occur in close physical and temporal proximity. These transitions must be carefully regulated to ensure that large numbers of spermatozoa are produced continuously throughout reproductive life. We address the question of how these transitions are coordinated at the cellular and molecular level.

During spermatogenesis, four key transitions stand out: (i) differ- entiation of spermatogonia, (ii) meiotic initiation, (iii) initiation of spermatid elongation, and (iv) release of spermatozoa into the lumen of seminiferous tubules (Fig. 1). In mice, spermatogonia begins with undifferentiated type A spermatogonia, which include the stem cells (1–4). Undifferentiated spermatogonia periodically undergo spermatogonial differentiation (also known as the A1–A2 transition) to become differentiating spermatogonia (also known as A2/A3/A4/A5/intermediate/B spermatogonia). During spermatogonial differentiation, the spermatogonia lose the capac- ity for self-renewal (5) and begin a series of six transit-amplifying mitotic divisions (6). Germ cells then become spermatocytes and undergo meiotic initiation (7, 8). DNA replication and two cell divisions follow, resulting in the formation of haploid, round spermatids, which elongate their nuclear and cytoplasmic contours to become spermatozoa. Finally, these spermatozoa are released into the lumen of seminiferous tubules.

The four key transitions of spermatogenesis are precisely coordinated in time and space and occur in close physical and tem- poral proximity, cyclically, with an 8.6-d periodicity in mice (9). The mouse testis is composed of structures known as seminiferous tubules (Fig. S1 A and B); within tubule cross-sections, one sees stereotypical collections or associations of germ cells at various steps of differ- entiation (Fig. 1). The precise coordination of these steps is called the “cycle of the seminiferous epithelium” (or “seminiferous cy- cle”). In mice, researchers have characterized 12 distinct cellular associations, known as seminiferous stages I to XII (10); the four key transitions all occur in stages VII and VIII (10, 11) (Fig. 1 and Fig. S1 B–D). This intimate proximity of the four transitions to each other is largely conserved in other mammals, including humans (12), rats (13, 14), hamsters (15), and rams (15). The layered genera- tions of germ cells in the seminiferous tubule are embedded in and supported by somatic (Sertoli) cells that supply factors essential for spermatogenesis (16). We sought to investigate the cooccurrence of the four key transitions, to understand the regulation of these transitions and the overall organization of spermatogenesis.

Both of the premeiotic transitions—spermatogonial differentia- tion and meiotic initiation—require retinoic acid (RA), a derivative

Significance

Male mouse sex cells mature into sperm through a 35-d process punctuated by four transitions, two occurring before meiosis (spermatogonial differentiation and meiotic initiation) and two after meiosis (spermatid elongation and sperm release). The four transitions occur in proximity spatially and temporally, with an 8.6-d periodicity. We describe how this coordination is achieved. The premeiotic transitions were known to be regulated by retinoic acid (RA). We show that RA also regulates the two postmeiotic transitions. RA levels change periodically, and meiotic cells contribute to its production. The two postmeiotic transitions require RA from meiotic cells while the premeiotic transitions require RA from somatic cells. These elements under- pin the spatiotemporal coordination of spermatogenesis to ensure constant sperm production throughout adult life.

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of vitamin A. In vitamin A-deficient (VAD) mice and rats, most germ cells arrest as undifferentiated spermatagonia (17, 18). In VAD rat testes, some germ cells also arrest just before meiosis, as preleptotene spermatocytes (19). When VAD animals are given RA or vitamin A, the arrested spermatogonia differentiate (17, 18). Furthermore, deficiency of the RA target gene Stra8 (20) and administration of vitamin A, the arrested preleptotene spermatocytes—containing aligned spermatozoa was increased (Fig. 2 B–D, Fig. S2 B–E, and SI Results); release of spermatozoa was inhibited not only in late stage VIII but also in stages IX and X (Fig. 2E and Fig. S2F). If this inhibition of spermatozoa release is a consequence of RA depletion, then injection of RA might be expected to induce premature release of spermatozoa. Indeed, at 2 d after a single RA injection, the percentage of tubules containing aligned spermatozoa was decreased, particularly in stage VIII (Fig. 2 B–D and Fig. S2 B–E). We conclude that RA induces the release of spermatozoa.

RA Promotes Initiation of Spermatid Elongation. We then tested whether RA also affects spermatid elongation, the other key postmeiotic transition (Fig. 1). In the unperturbed testis, spermatid development has been subdivided into 16 steps; step 7 and 8 round spermatids initiate their elongation in stage VIII and become step 9 elongating spermatids in stage IX (Fig. 1). If RA induces this initiation of spermatid elongation, then WIN18,446 injection should increase the percentage of seminiferous tubules that contain aligned spermatozoa, which are located along the luminal edge of the seminiferous tubules and are awaiting release into the lumen (Fig. S1D). Indeed, we confirmed that RA concentrations in testes were markedly increased 1 d after RA injection and markedly decreased after 2 or 4 d of daily WIN18,446 injection (Fig. 2D). In the unperturbed testis, spermatozoa are released in late stage VIII (Fig. 1). If RA induces this release, then WIN18,446 injection should increase the percentage of seminiferous tubules that contain aligned spermatozoa. RA Promotes Release of Spermatozoa. We chemically manipulated RA levels in living testes to probe whether RA induces the release of spermatozoa. To ensure the efficacy of our experimental protocols, we first confirmed that i.p. injection of exogenous RA raises the concentration of RA in testes, and, conversely, that injection of WIN18,446, an inhibitor of RA synthesis (30), lowers RA levels in testes. We monitored RA levels in testes both indirectly, by immunostaining for STRA8 as an RA-responsive marker (20, 31, 32), and directly, by measuring RA levels via liquid chromatography/mass spectrometry (LC/MS). In control testis sections, STRA8 protein was not detectable in germ cells in seminiferous stages V and VI (before key transitions) but was clearly present in spermatogonia and preleptotene spermatocytes in stages VII and VIII (during key transitions) (22) (Fig. S2A). As expected, at 1 d after a single RA injection, STRA8 was strongly induced in stages V and VI. Conversely, after 2 d of daily WIN18,446 injections, STRA8 was not expressed in stages VII and VIII. LC/MS assays confirmed that RA concentrations in testes were markedly increased 1 d after RA injection and markedly decreased after 2 or 4 d of daily WIN18,446 injection (Fig. 2A).

We next used pulse-chase labeling (33, 34) to confirm that RA causes spermatid elongation to be initiated earlier than it would normally occur. When a single BrdU injection is given, BrdU is incorporated into cells in mitotic or postmeiotic S phase. At 4 h after BrdU injection, the most advanced BrdU-labeled germ cells are
preleptotene spermatocytes that have undertaken premeiotic S and thereby initiated the meiotic program (Fig. S3 B and C). In the unperturbed tests, these BrdU-labeled preleptotene spermatocytes develop into step 8 round and step 9 and 10 elongating spermatids, respectively, 17.2 and 18.2 d after BrdU injection (Fig. 3C). We predicted that RA or WIN18,446 treatment would accelerate or delay spermatid development, respectively, at 18.2 d after the BrdU labeling, when the most advanced BrdU-labeled germ cells in unperturbed testes have initiated spermatid elongation. To test this, we gave mice a single BrdU injection, followed by daily RA or WIN18,446 injection during spermatid development (Fig. 3C), and harvested the testes at 17.2 d (Exps. 1 to 3) or 18.2 d (Exps. 4 to 6). We found that, at 17.2 d after BrdU injection, the most advanced BrdU-labeled germ cells were step 8 round spermatids in control tests, and there was no acceleration or delay under RA or WIN18,446 treatment (Fig. S3 D and F). This demonstrates that RA does not affect the development of round spermatids, from step 1 to step 8. However, as predicted, at 18.2 d after BrdU injection, the most advanced BrdU-labeled germ cells, which were step 10 spermatids in controls, had developed further—into step 11 spermatids—in RA-treated testes whereas they were still step 9 spermatids in WIN18,446-treated testes (Fig. 3D and Fig. S3F). Taken together, these findings provide strong evidence that RA promotes the initiation of spermatid elongation.

RA Has No Discernible Effect on Meiotic Progression. Given that four of the five generations of spermatogenic cells undergo transitions coregulated by RA in stages VII and VIII (Fig. 1), we wondered whether the remaining cell type at these stages, pachytene spermatocytes, also undergoes RA-regulated development during meiotic progression.

We examined this possibility by pulse–chase labeling. We gave mice a single injection of BrdU, followed by daily RA or WIN18,446 injection during meiotic progression (Fig. 3C, Exps. 7 to 9), and harvested the testes at 12.6 d, when, in control tests, the most advanced BrdU-labeled germ cells have completed meiotic progression and become step 1 and 2 spermatids. At 12.6 d, there was no acceleration or delay under RA or WIN18,446 treatment (Fig. S3 E and F). These results indicate that RA does not affect meiotic progression as assayed histologically. To verify that RA does not affect meiotic progression at the molecular level, we tested for key molecular features of meiotic progression by immunohistochemistry. We first gave mice a single injection of 5-ethyl-2′-deoxyuridine (EdU), followed by daily RA or WIN18,446 injection during meiotic progression (Fig. 4A), and harvested testes at 8.6 d (Exps. 10 to 12) and 11 d (Exps. 13 to 15), when in control testes the most advanced EdU-labeled germ cells have progressed to late pachytene stage and diplotene stage, respectively (35). We then immunostained the EdU-labeled nuclear spreads of spermatocytes for markers of meiotic prophase to distinguish among middle pachytene, late pachytene, and diplotene stages. Specifically, we used antibodies against phosphorylated H2A histone family member X (pH2AX) (a marker of DNA double strand breaks and sex body formation) (36), synaptonemal complex protein 3 (SYCP3) (a marker of synaptonemal complex (37), tests-specific histone (H1t) (a marker of middle/late pachytene stages) (38), and ataxia telangiectasia and Rad3-related protein (ATR) (a marker of DNA repair and sex body formation) (39). Based on these markers (SI Results), at 8.6 d and 11 d after EdU injection, the most advanced EdU-labeled germ cells in control testes were, respectively, late pachytene and diplotene spermatocytes, and there was no acceleration or delay after RA or WIN18,446 treatment (Fig. 4B–D and Fig. S4). Importantly, expression and localization of all of the meiotic markers appeared to be unaffected by either RA or WIN18,446 treatment. We conclude that RA has no discernible effect on meiotic progression from leptotene stage to diplotene stage, including the development of pachytene spermatocytes.

Our results demonstrate that RA regulates release of spermatooza and initiation of spermatid elongation, as well as spermatogonial differentiation and meiotic initiation, but not meiotic progression, in stages VII and VIII. To explore the mechanism by which these four transitions are coordinated in time and space by RA, we next focused on changes in RA levels in the seminiferous tubules.
In unperturbed mouse testes, the seminiferous stages of the tubules are not synchronized across the testis but instead are highly diverse (40) (Fig. S1B). When mice are treated daily with WIN18,446 from postnatal day 2 (P2), germ cells arrest as undifferentiated spermatogonia at stages VII and VIII (41) (Fig. 1). After a single injection of RA into these WIN18,446-pretreated mice, the arrested germ cells undergo spermatogonial differentiation to initiate spermatogenesis in a synchronized manner (41). Thus, we gave daily injections of WIN18,446 from P2 until P14 (Fig. S5A and SI Results) and then injected a single dose of RA at P15, harvesting testes 5, 8, 9, or 10 d after the RA injection (Fig. S4 and Fig. S5B). As we anticipated, at 5 d after RA injection, when seminiferous tubules were synchronized at stages II to IV, the RA concentration was negligible or very low (Fig. SB and Fig. S5D). Then, at 8 d, in stages VII to IX, the RA concentration was significantly increased, and it remained high at 9 d, in stages IX to XI, before falling modestly at 10 d, in stages XI to I (difference between RA levels in stages VII to IX and XI to I is not significant; P > 0.05, Tukey–Kramer test). We conclude that RA concentrations change periodically, as also reflected by, and in good agreement with, STRA8 expression patterns (Fig. S5C).

**RA Concentration Increases When Aldh1a2-Expressing Pachytene Spermatocytes First Appear.** We then considered the identity of the cells within the seminiferous tubules that produce RA, which have been shown to express an RA-synthesizing enzyme, aldehyde dehydrogenase 1A1 (Aldh1a1) (42, 43), are likely the source of the RA measured in the experiments just described. Another RA-synthesizing enzyme, Aldh1a2, is abundantly expressed in pachytene spermatocytes and their descendants, diploptene spermatocytes, from stages VII through XII (42, 43). If pachytene spermatocytes actually produce RA from stage VII onward, then RA levels in the testes of young males should rise shortly after these Aldh1a2-expressing pachytene cells first appear (at about 15 d after RA-induced spermatogonial differentiation). To test this prediction, we harvested stage-synchronized testes at 12, 15, 16, and 17 d after RA injection, when pachytene and then diploptene spermatocytes first appear (Fig. S4 and Fig. S5B), for absolute quantification of RA levels. At 12 d after RA injection, when seminiferous tubules containing pachytene spermatocytes were in stages II to IV, RA concentration was very low, similar to 5 d after RA injection (Fig. SB and Fig. S5D). As predicted, at 15 d after RA injection, in stages VII to IX, RA concentration increased and significantly higher than at 8 d, when pachytene spermatocytes were not yet present. The higher RA concentration was maintained at 16 d and 17 d after RA injection, when pachytene to stage IX to XI and stages XI to I, respectively. The simplest interpretation of these data is that pachytene spermatocytes not only express Aldh1a2 but also produce RA from stages VII and VIII onward.

**Depletion of Pachytene Spermatocytes Reduces RA Concentration in Adult Testes.** Building upon these findings, we hypothesized that RA produced by pachytene spermatocytes might induce or contribute to some of the key germ cell transitions that occur in stage VII and VIII tubules (Fig. 1). To explore the functional roles of RA produced by pachytene spermatocytes, we conditionally removed pachytene spermatocytes from adult testes, using a cell depletion assay. Hydroxyurea (HU) specifically inhibits the ribonucleotide reductase that generates deoxyribonucleotides required for DNA replication, by binding the enzyme’s iron molecules, thereby killing cells that are in S phase (44, 45). We injected adult mice with HU six times, at 6-h intervals, and we first assessed the results of HU treatment at 36 h (1.5 d) after the initial injection. As expected, all differentiating spermatogonia (A1/A2/A3/A4/intermediate/B spermatogonia) and stage VII preleptotene spermatocytes had been eliminated by HU treatment (Fig. 5C, Fig. S6A, and SI Results), with no cytotoxic effects on the mature germ cells, or on Sertoli cells (46). At 11.6 d after the first HU injection, the specific cohort of germ cells that normally would have transited to pachytene and diploptene in stages I to XII was missing from the testis, as expected (Fig. 5C and Fig. S6B); in stage VII and VIII tubules, pachytene spermatocytes were the...
only missing cells, again as expected (Fig. 5D and Fig. S6C). Importantly, testicular RA concentrations were significantly lower than in controls (Fig. 5E and Fig. S6D and E). Also, as predicted, we observed that the remaining levels of RA, which probably derived from Sertoli cells, were eliminated by coinjection of WIN18,446 with HU. We conclude that conditional depletion of pachytene spermatocytes reduces RA levels in adult testes.

To confirm that the reduced RA concentration at 11.6 d after the first HU injection was associated with the depletion of a specific RA-synthesizing enzyme from seminiferous tubules, we carried out single-molecule fluorescence in situ hybridization (smFISH). Because smFISH probes detect and localize each target mRNA molecule as a punctate signal, these signals can be quantified to determine the number of transcripts per cell (47) (SI Results). In control stage VII and VIII tubules, Aldh1a2 was expressed specifically in pachytene spermatocytes, Aldh1a1 was expressed specifically in Sertoli cells, and Aldh1a3 was expressed at very low levels, if at all, in germ cells and Sertoli cells (Fig. 6), all as previously reported (42, 43); we used kidney as a positive control for Aldh1a2 transcripts (unpublished results). As predicted, at 11.6 d after the first HU injection, Aldh1a2 transcripts were dramatically reduced in seminiferous tubules, likely because of the absence of pachytene spermatocytes. Importantly, we observed no compensatory up-regulation of Aldh1a1, Aldh1a2, or Aldh1a3 in the remaining germ or Sertoli cells of the seminiferous tubules, despite the fact that such compensation has been observed to accompany genetic ablation of these genes in other contexts (48, 49). Conversely, HU depletion of pachytene spermatocytes did not result in reduced expression of Aldh1a1, Aldh1a2, or Aldh1a3 in the remaining germ or Sertoli cells. Thus, the reduction in RA concentration upon depletion of pachytene spermatocytes was not due to reduced expression of RA-synthesizing enzymes in the remaining cells of the seminiferous tubules. Instead, depletion of ALDH1A2-expressing pachytene spermatocytes reduces testicular RA concentrations directly. We then proceeded to test whether RA from pachytene spermatocytes contributes functionally to the four key transitions in stages VII and VIII, using HU-treated testes.

RA from Pachytene Spermatocytes Is Required for Postmeiotic, but Not for Premeiotic, Transitions. If RA from pachytene spermatocytes is required for release of spermatozoa and initiation of spermatid elongation, seminiferous tubules should display accumulations of aligned (unreleased) spermatozoa and round spermatids at 11.6 d after the first HU injection. Indeed, at 11.6 d after the first HU injection, seminiferous tubules displayed increased numbers of aligned germ cells in nuclear spreads of spermatocytes (Fig. 7). Acrosome reactions after entry into meiotic prophase. (A) Diagram of predicted development of most advanced EdU-labeled germ cells following single EdU injection. Mice received daily injections of RA or WIN18,446 until the observation point, when testes were harvested (Exps. 10 to 15). D, diploptene spermatocytes; αP, early pachytene spermatocytes; L, leptotene spermatocytes; IP, late pachytene spermatocytes; mP, mid pachytene spermatocytes; Pl, preleptotene spermatocytes; Z, zygotene spermatocytes. (B and C) The most advanced EdU-labeled germ cells in nuclear spreads of spermatocytes in control or daily RA- or WIN18,446-injected mice at 8.6 d (B; Exps. 10 to 12); late pachytene stage or 11 d (C; Exps. 13 to 15; diploptene stage) after a single EdU injection, immunostained for SYCP3 (green), γH2AX (red), and EdU (blue). (B, Far Left) Representative mid-pachytene spermatocyte showing no EdU signal in controls. (Scale bars: 10 μm.) (D) Numbers and percentages of mid-pachytene, late pachytene, and diploptene cells positive for EdU. Percentages of EdU+ cells did not differ significantly among control, RA-treated, and WIN-treated groups (three biological replicates; P > 0.05; χ² test).

**Fig. 4.** RA has no discernible effect on meiotic progression after entry into meiotic prophase.
controls (Fig. 6A and Fig. S7D), suggesting that these germ cells are responding normally, to endogenous RA, even in the absence of pachytene spermatocytes and the RA that they provide. If RA from pachytene cells is not required for meiotic initiation, then premeiotic or early meiotic (leptotene) spermatocytes should not be increased or decreased after pachytene spermatocyte depletion. Indeed, at 11.6 d after the first HU injection, there was no increase in the numbers of tubules containing premeiotic (preleptotene) spermatocytes or decrease in the numbers of tubules containing early meiotic (leptotene) spermatocytes (Fig. 7C and Fig. S8 A, B, E, and F). Similarly, we confirmed that genetic depletion of pachytene spermatocytes, in Dmc1- or Spo11-deficient mice, had no discernible effect on preleptotene or leptotene spermatocytes; in these mutant mice, defects in meiotic progression result in apoptosis of pachytene spermatocytes before or during stage IV (50–53). By contrast, coinjection of WIN18,446 with HU did increase preleptotene spermatocytes and decrease leptotene spermatocytes, and coinjection of RA with HU decreased preleptotene spermatocytes and increased leptotene spermatocytes. These results suggest that meiotic initiation requires RA from sources other than pachytene spermatocytes.

Finally, we tested whether RA from pachytene spermatocytes is required for spermatogonial differentiation, by immunostaining for functional markers of spermatogonial differentiation, STRA8 and KIT (22, 54). As mentioned above, at 11.6 d after the first HU injection, type A spermatogonia in stages VII and VIII of control (Left) or HU-injected (Right) mice, stained with hematoxylin and periodic acid-Schiff (He-PAS). Orange dots, pachytene spermatocytes. Scale bar: 30 μm. (E) Quantification of RA levels (pmol/g of testes) in control and HU-injected adult mice. HU + WIN, mice were given injections of HU and then daily injections of WIN18,446, beginning 7.6 d after first HU injection. WIN, mice received daily injections of WIN18,446 for 4 d. Dashed line, limit of detection. ND, not detected (below limit of detection). The far right “WIN” bar graph shows results of two biological replicates. Error bars, mean ± SD, *P < 0.01 (Tukey–Kramer test).
Discussion

RA Regulates Four Key Transitions of Spermatogenesis. We investigated the chemical basis of the exquisite coordination of spermatogenesis, the process by which diploid spermatogonia differentiate into specialized haploid gametes, spermatozoa, at a constant rate. We found that rising levels of an extrinsic signal, RA, regulate four key developmental transitions in mouse spermatogenesis: spermatogonial differentiation and meiotic initiation before meiosis (22) and initiation of spermatid elongation and release of spermatozoa after meiosis (Fig. 8 A). We also found that these pre- and postmeiotic transitions require RA from different cell sources: Sertoli (somatic) cells and pachytene spermatocytes (meiotic cells), respectively (Fig. 8 B). We will now discuss the implications of these findings for understanding the regulation of this temporally and spatially coordinated system of cell division and differentiation (10, 14).

By chemically manipulating the levels of RA in the testes of live animals, we found that RA plays critical, primary roles in the two postmeiotic transitions: initiation of spermatid elongation and release of spermatozoa. Indeed, both of these transitions were inhibited within 2 d after starting injections of a potent inhibitor of RA synthesis, WIN18,446. Conversely, injection of RA was sufficient to induce both of these postmeiotic transitions, again within 2 d. These findings align with, and help explain, prior reports of postmeiotic defects caused by genetic or chemical ablation of RA receptors (RARs) or RA-synthesizing enzymes (23–29). Further, we found that, after meiotic initiation, progression and completion of meiosis and subsequent development of round spermatids are unaffected by manipulations of RA levels, suggesting that RA plays little or no direct role in this middle, 17-d stretch of spermatogenesis. Because RA also coregulates the two premeiotic transitions (17–19, 22), our present findings demonstrate that four of the five generations of spermatogenic cells present in stage VII and VIII tubules undergo transitions regulated by a single extrinsic cue, RA.

It remains to be determined whether RA acts directly on germ cells or indirectly, via Sertoli cells, to regulate the two postmeiotic transitions. RA serves as a ligand to nuclear receptors known as RARs and retinoid X receptors (RXRs), which bind to RA response elements (RAREs) in the regulatory regions of target genes (55). These receptors are expressed specifically in round spermatids (42), suggesting that RA acts directly on round spermatids to induce their elongation. Indirect RA signaling, via RARs/RXRs in Sertoli cells (42), may also contribute...
Recent studies have addressed the question of whether RA levels fluctuate in the testis to regulate the 8.6-d cycle of spermatogenesis. Insights into the question have emerged from studies of Aldh1a gene expression, RAR receptor function, and relative measurements of RA levels (27, 43, 57). However, the actual concentrations of RA in testes, and their dynamics and range, remained unclear. We previously reported that the RA-responsive marker STRA8 is expressed periodically in the unperturbed testis, and, on this basis, we postulated that RA levels rise and fall periodically (22). By absolute quantification of RA levels, we now provide direct evidence that RA levels change periodically and that these changes are mirrored by changes in STRA8 expression: RA levels are low in stages II to VI, rise in stages VII and VIII, and remain high until stages XII/I. This long elevation of RA levels in stages VII to XII/I is consistent with other published expression data and functional studies (27, 42, 43). By contrast, Hogarth et al. (57) suggested, based on relative RA measurement, a sharp peak in RA levels in stages VIII and IX. This difference between Hogarth et al.’s findings and those reported here likely reflects differences in experimental designs and in methods of measurement and analysis employed. For example, while we analyzed stage-synchronized testes between 5 and 17 d after RA injection, Hogarth et al. analyzed testes between 42 and 50 d after RA injection, by which point synchrony may be less consistent (41, 58).

Our findings demonstrate that, in the unperturbed testis, RA concentrations rise in stages VII and VIII, thereby coordinating four spermatogenic transitions, and they remain elevated until stages XII/I. We previously showed that germ cells have stage-limited competencies to undergo spermatogonial differentiation and meiotic initiation (22). These windows of competence begin while RA levels are low, and end (in stage VIII) while RA levels are still high, so that the competencies intersect with high RA levels briefly to confine the timing of premeiotic transitions (to stages VII and VIII). It remains to be determined whether round spermatids have a similarly limited competency (ending in stage VIII) to undertake the initiation of spermatid elongation (in stages VII and VIII).

**Periodic RA from Sertoli Cells and Pachytene Spermocytes Coordinates Four Transitions.** Based on the observation that an RA-synthesizing enzyme, *Aldh1a2*, is expressed in pachytene spermatocytes (42, 43) and that spermatogonial differentiation requires RA, Sugimoto et al. (43) hypothesized that production of RA by pachytene spermatocytes induces the next round of spermatogonial differentiation. Indeed, we found that RA concentrations increase when *Aldh1a2*-expressing pachytene spermatocytes first appear in young males (Fig. 5B), consistent with Sugimoto et al.’s suggestion that pachytene spermatocytes produce RA. However, using genetic and chemical cell depletion assays, we showed that RA from pachytene spermatocytes is required for postmeiotic transitions, but not for premeiotic transitions. Based on the findings reported here, we propose that pre- and postmeiotic transitions are regulated by RA from different sources. Specifically, the model posits that Sertoli cells periodically produce RA to regulate two premeiotic transitions and that pachytene spermatocytes produce RA to regulate two postmeiotic transitions. Several observations support the model that these premeiotic transitions are coordinated by RA from Sertoli cells (but not from pachytene spermatocytes). First, Sertoli cell-specific ablations of *Aldh1a1-3* cause an arrest of the first spermatogonial differentiation in postnatal mice (28). Second, using RA quantification, we now show that RA periodicity (with rising RA concentrations in stages VII and VIII) is maintained even in the absence of pachytene spermatocytes. Further, we provide both chemical and genetic evidence that depletion of pachytene spermatocytes does not affect the timing of the premeiotic transitions: These timed transitions are maintained in *Dmc1-* and *Spo11*-deficient males, in which pachytene spermatocytes are

**RA Concentrations Change Periodically, as Reflected by STRA8 Expression Patterns.** Recent studies have addressed the question of whether RA levels fluctuate in the testis to regulate the 8.6-d cycle of spermatogenesis. Insights into the question have emerged from studies of Aldh1a gene expression, RAR receptor function, and relative measurements of RA levels (27, 43, 57). However, the actual concentrations of RA in testes, and their dynamics and range, remained unclear. We previously reported that the RA-responsive marker STRA8 is expressed periodically in the unperturbed testis, and, on this basis, we postulated that RA levels rise and fall periodically (22). By absolute quantification of RA levels, we now provide direct evidence that RA levels change periodically and that these changes are mirrored by changes in STRA8 expression: RA levels are low in stages II to VI, rise in stages VII and VIII, and remain high until stages XII/I. This long elevation of RA levels in stages VII to XII/I is consistent with other published expression data and functional studies (27, 42, 43). By contrast, Hogarth et al. (57) suggested, based on relative RA measurement, a sharp peak in RA levels in stages VIII and IX. This difference between Hogarth et al.’s findings and those reported here likely reflects differences in experimental designs and in methods of measurement and analysis employed. For example, while we analyzed stage-synchronized testes between 5 and 17 d after RA injection, Hogarth et al. analyzed testes between 42 and 50 d after RA injection, by which point synchrony may be less consistent (41, 58).

Our findings demonstrate that, in the unperturbed testis, RA concentrations rise in stages VII and VIII, thereby coordinating four spermatogenic transitions, and they remain elevated until stages XII/I. We previously showed that germ cells have stage-limited competencies to undergo spermatogonial differentiation and meiotic initiation (22). These windows of competence begin while RA levels are low, and end (in stage VIII) while RA levels are still high, so that the competencies intersect with high RA levels briefly to confine the timing of premeiotic transitions (to stages VII and VIII). It remains to be determined whether round spermatids have a similarly limited competency (ending in stage VIII) to undertake the initiation of spermatid elongation (in stages VII and VIII).

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depleted. Finally, Sertoli cells establish a stage-dependent cycle of metabolism in neonatal males, before pachytene spermatocytes first appear (59), leading us to propose that Sertoli cells may produce RA periodically to coordinate the premeiotic transitions, independent of RA from pachytene spermatocytes.

We do not yet know why the pre- and postmeiotic transitions require different cellular sources of RA. In the seminiferous tubule, premeiotic germ cells are confined to the basal compartment, while pachytene spermatocytes and postmeiotic germ cells are located, by tight junctions formed between Sertoli cells (60) (Fig. 8 B). This basal vs. luminal arrangement may influence the diffusion, degradation, and availability of RA produced by Sertoli cells and pachytene spermatocytes. However, we surmise that RA is not segregated strictly between basal and luminal compartments. Indeed, in Sertoli cell-specific Aldh1a1−/− deficient testes, the resulting arrest at the first (juvenile) spermatogonial differentiation could be rescued by a single injection of RA, with all germ cell layers observed subsequently, in adult testes (28). This suggests that RA from pachytene spermatocytes can diffuse into the basal compartment to affect the two premeiotic transitions, perhaps redundantly with RA from Sertoli cells. Conversely, 20 wk after RA injection, Sertoli cell-specific Aldh1a1−/− deficient males displayed abnormalities in release of spermatids (28), suggesting that Sertoli cell production of RA may contribute, modestly, to this process. Thus, pachytene spermatocytes may work collaboratively with Sertoli cells to ensure high RA concentrations throughout the seminiferous tubules. Alternatively, the postmeiotic transitions may simply require a higher level of RA (from Sertoli cells plus pachytene spermatocytes) than the premeiotic transitions.

In summary, we conclude that both Sertoli cells and pachytene spermatocytes produce RA to establish a periodicity of testicular RA levels, which coordinate the four key transitions in spermatogenesis in close physical and temporal proximity. Our findings may have practical implications for in vitro spermatogenesis. Recently, successful spermatogenesis from ES cells to haploid spermatid-like cells was achieved by culturing within seminiferous tubules. Alternatively, the postmeiotic transitions may simply require a higher level of RA (from Sertoli cells plus pachytene spermatocytes) than the premeiotic transitions.

In vitro (to produce functional spermatozoa) has yet to be achieved. Our findings that haploid spermatids require periodic RA signals to complete spermatogenesis may help advance the technology of in vitro gamete production.

**Materials and Methods**

**Mice.** Three types of mice were used: WT (C57BL/6NTac), Dmc1−/−deficient (B6. Cg-Dmc1tm1trc/J) (50), and Spodf−/−deficient (B6-129X1 Spo11tm20Min) (52). 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.** Unless otherwise noted, data are represented as mean ± SD or SE of three or more biological replicates. When comparing two groups, the t test (one-tailed as indicated) was employed. When comparing three or more groups, a one-way ANOVA with the Tukey-Kramer post hoc test was used.

**Histology.** Testes were fixed overnight in Bouin’s solution, embedded in paraffin, sectioned, and stained 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 (62). See SI Materials and Methods for details on identification of seminiferous tubule stages.

**Chemical Treatments.** Mice received i.p. or s.c. injections of all-trans RA, WIN18,446, BrdU, EdU, and/or hydroxyurea (HU). See SI Materials and Methods for details.

**Immunostaining on Testis Sections.** Testes were fixed overnight in either Bouin’s solution or 4% (wt/vol) paraformaldehyde (PFA), 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 blocked, incubated with the primary antibody, washed with PBS, and incubated with secondary antibody. Detection was fluorescent or colorimetric. See SI Materials and Methods for details.

**Nuclear Spreads of Spermatocytes.** Testes were dissected to obtain single cells, including spermatocytes, as previously described (8). Cell suspensions were placed on slides and fixed in 1% (wt/vol) PFA. Slides were washed, air dried, and stored at −80 °C before use. For immunostaining, slides were brought to room temperature and washed with PBS. See SI Materials and Methods for details.

**Single-Molecule Fluorescent in Situ Hybridization.** Probe design, synthesis, and coupling were as previously described (47, 63). Testes were fixed in 4% (wt/vol) PFA and embedded in optimal cutting temperature compound (O.C.T.). Frozen blocks were sectioned at 8 μm thickness and dehydrated overnight in 70% (vol/vol) ethanol at 4 °C. Hybridization was performed as previously described (47, 63). Counting of individual mRNA particles, image stitching, and data analysis were performed using custom Matlab software as previously described (47, 63). See SI Materials and Methods for details.

**Absolute Quantification of RA Levels.** Sample preparation and RA quantification were performed according to a published protocol (64). Testes were collected and homogenized by hand in ground glass homogenizers on ice in 1 mL of saline (0.9% NaCl). All-trans RA was extracted and quantified by liquid chromatography/mass spectrometry. See SI Materials and Methods for details.

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