

Spring 2020 – Systems Biology of Reproduction
Discussion Outline – Testis Systems Biology
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
February 27, 2020
Week 7

Testis Systems Biology

Primary Papers:

1. Endo, et al. (2015) PNAS E2347-2356
2. Yu, et al., (2019) Molecular Human Reproduction 25:507
3. Guo, et al. (2018) Cell Research 28:1141

Discussion

Student 16: Reference 1 above

- How do retinoids and Stra8 interact?
- What experimental design was used?
- What is the integration of retinoid actions and spermatogenesis?

Student 17: Reference 2 above

- What was the experimental design and culture system used?
- What spermatogenic process occurred in vitro?
- How could this technology be applied?

Student 18: Reference 3 above

- What is the experimental and systems approach?
- What single cell expression and epigenetic relationships exist?
- What insights are provided on testis cell biology?

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

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Contributed by David C. Page, March 24, 2015 (sent for review January 18, 2015; reviewed by William W. Wright)

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_{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.

Author contributions: T.E., K.A.R., E.L.A., D.G.d.R., and D.C.P. designed research; T.E., K.A.R., E.L.A., and A.E.B. performed research; T.E., K.A.R., E.L.A., and D.G.d.R. analyzed data; and T.E., K.A.R., and D.C.P. wrote the paper.

Reviewers included: W.W.W., Johns Hopkins Bloomberg School of Public Health.

The authors declare no conflict of interest.

Data deposition: The mRNA-sequencing dataset reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE67169).

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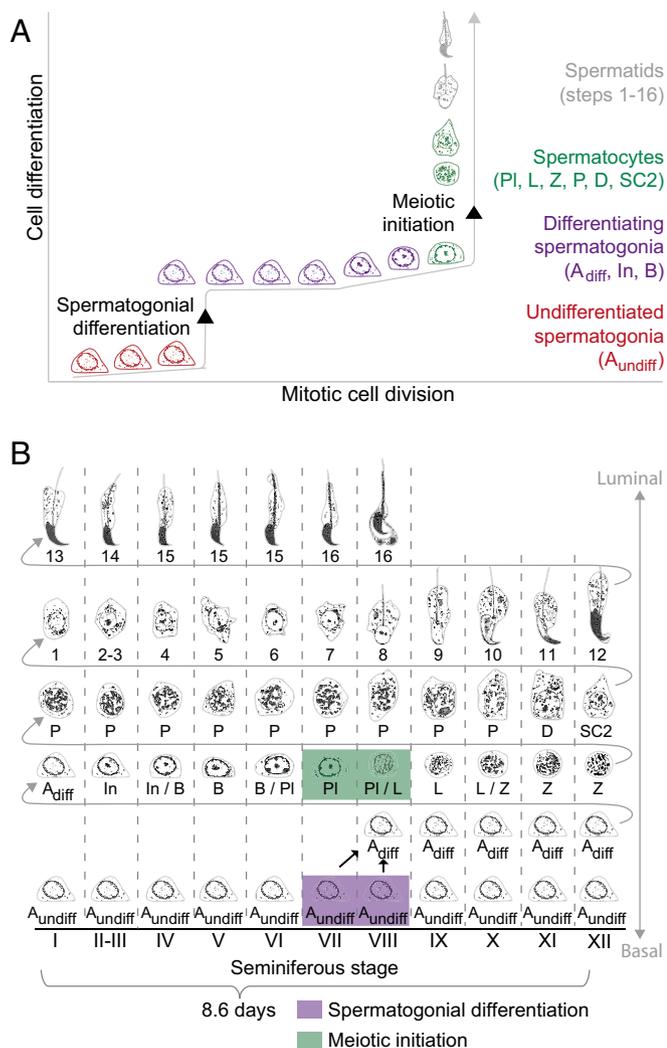


Fig. 1. Overview of spermatogenesis. (A) Diagram of mouse spermatogenesis. Germ cells develop from undifferentiated, mitotic spermatogonia (red, bottom left), to differentiating spermatogonia (purple, middle left), to spermatocytes (germ cells that undergo meiosis, green, middle right), to haploid spermatids (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

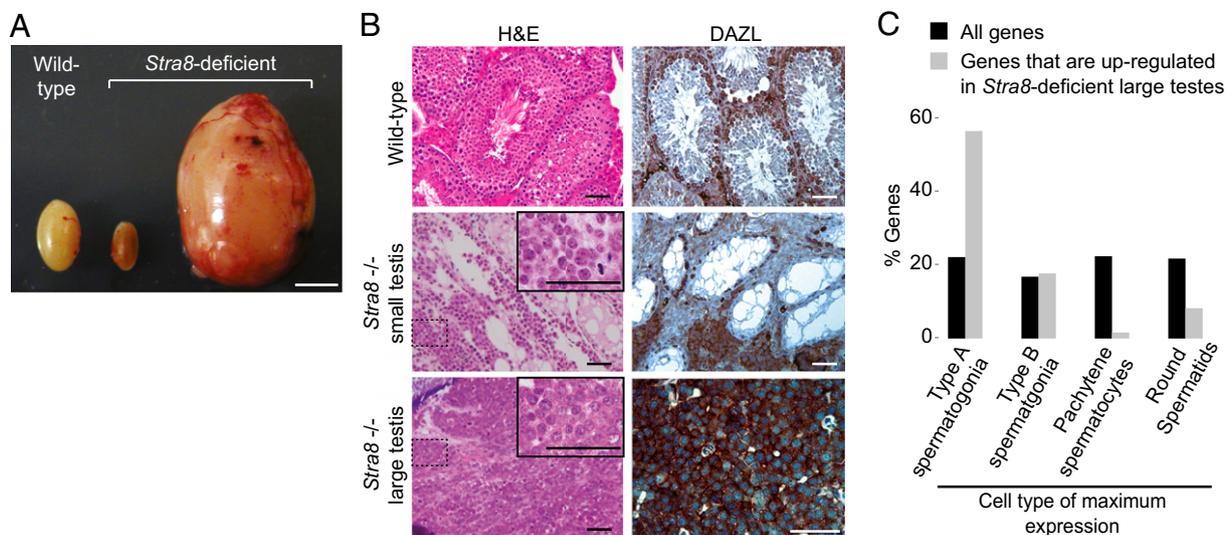


Fig. 2. Aged *Stra8*-deficient testes accumulate type A spermatogonia. (A) Wild-type (left) and *Stra8*-deficient small (center) and large (right) testes from 1-y-old mice. (Scale bar, 5 mm.) (B) Testis sections from 1-y-old mice: wild-type (Top), *Stra8*-deficient small testis (Middle), and *Stra8*-deficient 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-

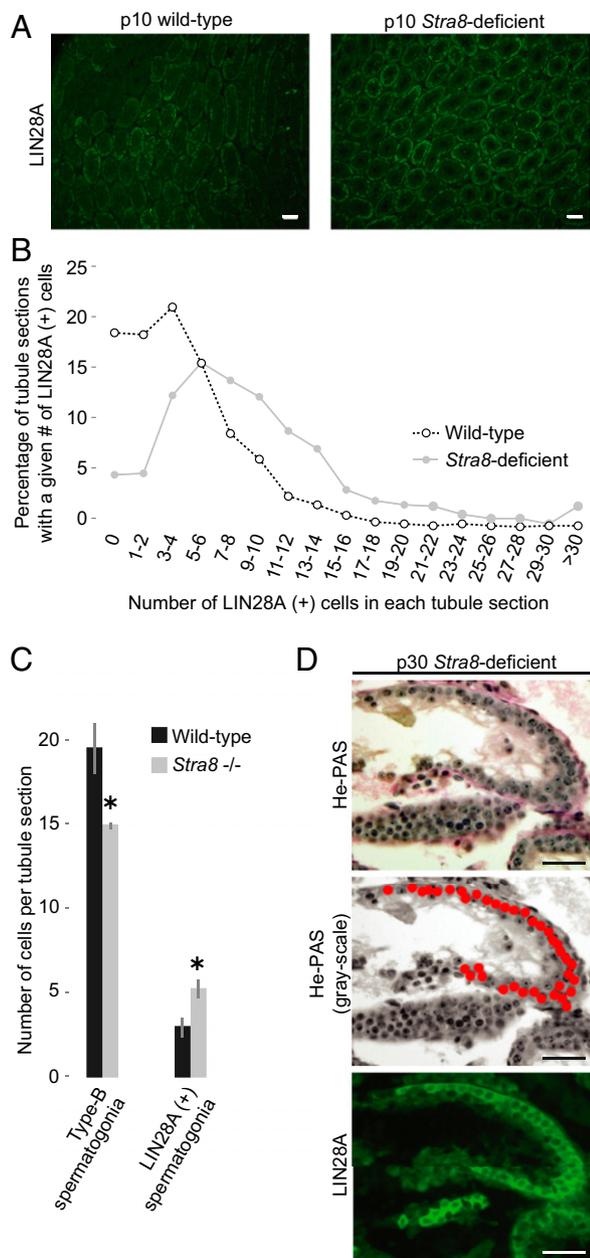


Fig. 3. Testes of young *Stra8*-deficient males are enriched for LIN28A-positive spermatogonia. (A) Immunostaining for LIN28A on postnatal day 10 (p10) testis sections: wild-type (Left) and *Stra8*-deficient (Right). (Scale bars, 50 μ m.) (B) Histogram of number of LIN28A-positive cells per tubule cross-section, in p10 wild-type and *Stra8*-deficient testes. For each genotype, testes from three mice were counted and averaged. (C) Number of type B and LIN28A-positive spermatogonia per tubule cross-section, in p30 wild-type and *Stra8*-deficient testes. Only tubules containing ≥ 1 type B spermatogonia were counted. Error bars, mean \pm SD * $P < 0.01$ (one-tailed Welch's *t* test). (D) Clusters of LIN28A-positive undifferentiated spermatogonia in p30 *Stra8*-deficient testes. (Top) Hematoxylin and periodic acid-Schiff (He-PAS) staining. (Middle) Grayscale version of top panel, with red dots indicating type A spermatogonia. (Bottom) LIN28A immunostaining on adjacent section. (Scale bars, 50 μ m.)

erous epithelium (hereafter referred to as “seminiferous stage”) (Fig. 1B and Fig. S1B). In the testis, particular germ-cell types are always found in the same tubule cross-section; Oakberg (33) identified 12 such stereotypical associations of germ cells, called stages I–XII. Any given section of a seminiferous tubule cycles through all 12 stages in order, with a full seminiferous cycle

encompassing these stages taking 8.6 d. Spermatogonial differentiation and meiotic initiation occur together in stages VII/VIII. Consistent with our hypothesis, STRA8 expression was rare in stages II–VI (before spermatogonial differentiation) then increased rapidly in stages VII–VIII (during spermatogonial differentiation) and remained high thereafter, in stages IX–I (Fig. S1C). [Similar increases in STRA8 expression in stage VII or VIII have been previously reported (34, 35)]. We further find that STRA8 expression overlapped with PLZF in stages VII–VIII and was limited to PLZF-low and -negative spermatogonia thereafter, in stages IX–X (Fig. 4A and B and Fig. S4A). We thus conclude that STRA8 expression begins in late undifferentiated spermatogonia and persists in differentiating spermatogonia.

Intriguingly, in stages VII–VIII, STRA8 expression increased in preleptotene spermatocytes (premeiotic cells) as well as in undifferentiated spermatogonia (Fig. S1D and E) (27, 35). Because STRA8 promotes spermatogonial differentiation and is required for meiotic initiation, precisely timed increases in STRA8 expression might coordinate both transitions, ensuring their cooccurrence in stages VII–VIII.

STRA8 expression is induced by RA (22, 23, 26, 28). We hypothesized that RA, acting through *Stra8*, coordinates spermatogonial differentiation with meiotic initiation. To test this, we perturbed RA signaling in the testes of wild-type mice, by injecting either RA or WIN18,446, an inhibitor of RA synthesis (36, 37). We first predicted that injected RA would induce ectopic STRA8 expression both in undifferentiated spermatogonia and in premeiotic cells (differentiating spermatogonia/preleptotene spermatocytes). Furthermore, injected RA should induce both precocious spermatogonial differentiation and precocious meiotic initiation. In contrast, WIN18,446 should inhibit STRA8 expression, spermatogonial differentiation, and meiotic initiation. We proceeded to test these predictions.

Injected RA Induces Precocious STRA8 Expression in Both Undifferentiated Spermatogonia and Premeiotic Germ Cells. We first verified that injected RA induced precocious STRA8 protein expression in the spermatogonial population, which encompasses both undifferentiated spermatogonia and premeiotic cells. In the unperturbed wild-type testis, spermatogonia began to express STRA8 in stages VII–VIII (during spermatogonial differentiation/meiotic initiation) (Fig. 4C). At 1 d after RA injection, STRA8 expression was strongly induced in spermatogonia in stages II–VI (Fig. 4C), as previously reported (34). In contrast, when we treated mice for 2 d with the RA synthesis inhibitor WIN18,446, spermatogonial STRA8 expression was almost completely eliminated in all stages (Fig. 4C and D).

We next showed that injected RA induced precocious STRA8 expression specifically in undifferentiated spermatogonia, by staining testis sections and intact testis tubules for STRA8 and PLZF (Fig. 4A, B, and E and Fig. S4A and B). Indeed, after RA injection, STRA8 expression was strongly induced in a subset of undifferentiated spermatogonia. STRA8 was not induced in any undifferentiated spermatogonia in stages IX–X, but only in late undifferentiated spermatogonia in stages II–VI. We conclude that a very specific subset of undifferentiated spermatogonia is competent to express STRA8.

Finally, we demonstrated that injected RA could induce precocious STRA8 expression in premeiotic cells, which we identified by nuclear morphology and by absence of PLZF expression (Fig. S4C). In the unperturbed testis, STRA8 was expressed in preleptotene spermatocytes in stages VII–VIII (during meiotic initiation) but was otherwise absent in premeiotic cells (Fig. 4F and Fig. S4D). After RA injection, STRA8 was strongly induced in all premeiotic cells, including intermediate and type B spermatogonia and preleptotene spermatocytes, in stages II–VI. We conclude that both undifferentiated spermatogonia and premeiotic cells precociously express STRA8 when exposed to RA in vivo.

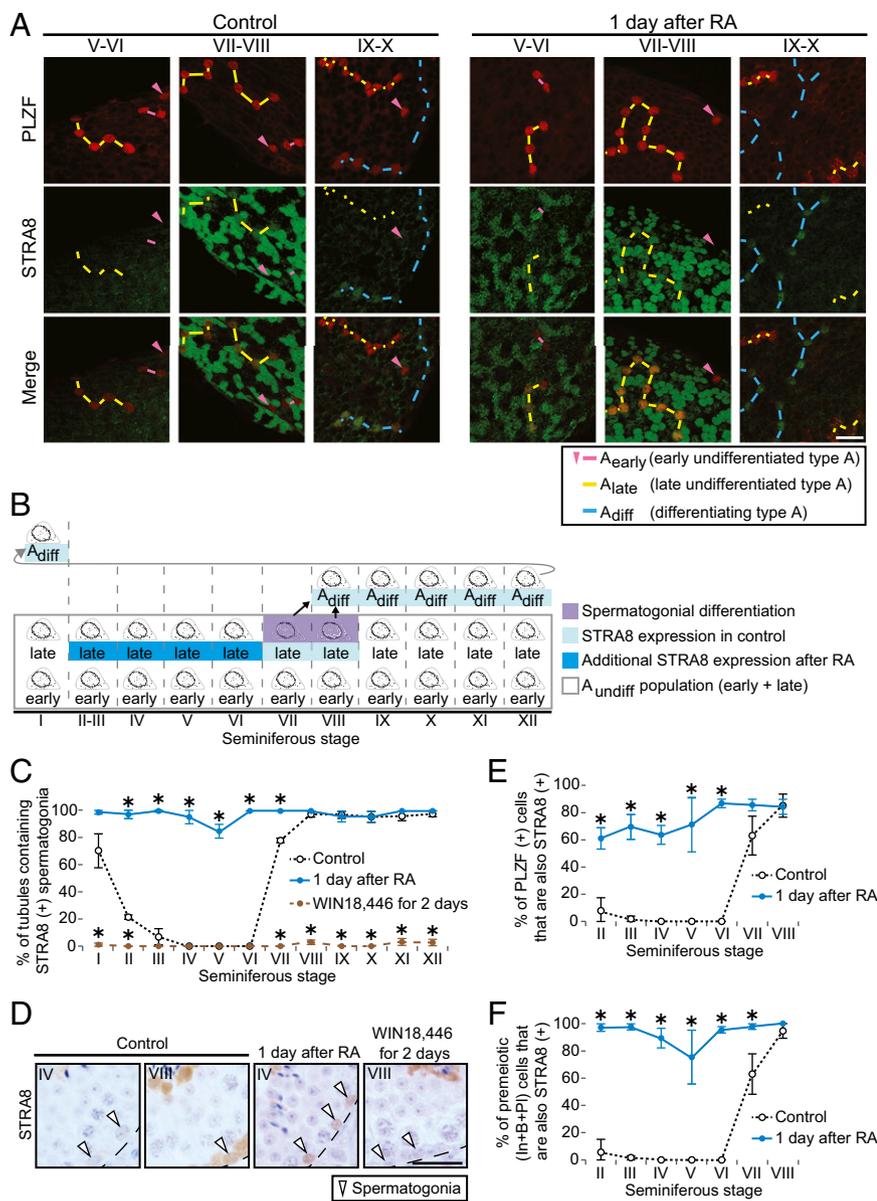


Fig. 4. STRA8 protein is normally present in late undifferentiated spermatogonia and can be precociously induced by injected RA. (A) Whole-mount immunostaining of intact wild-type testis tubules for PLZF (red) and STRA8 (green), in controls (Left) and 1 d after RA injection (Right). Arrowheads: isolated (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)

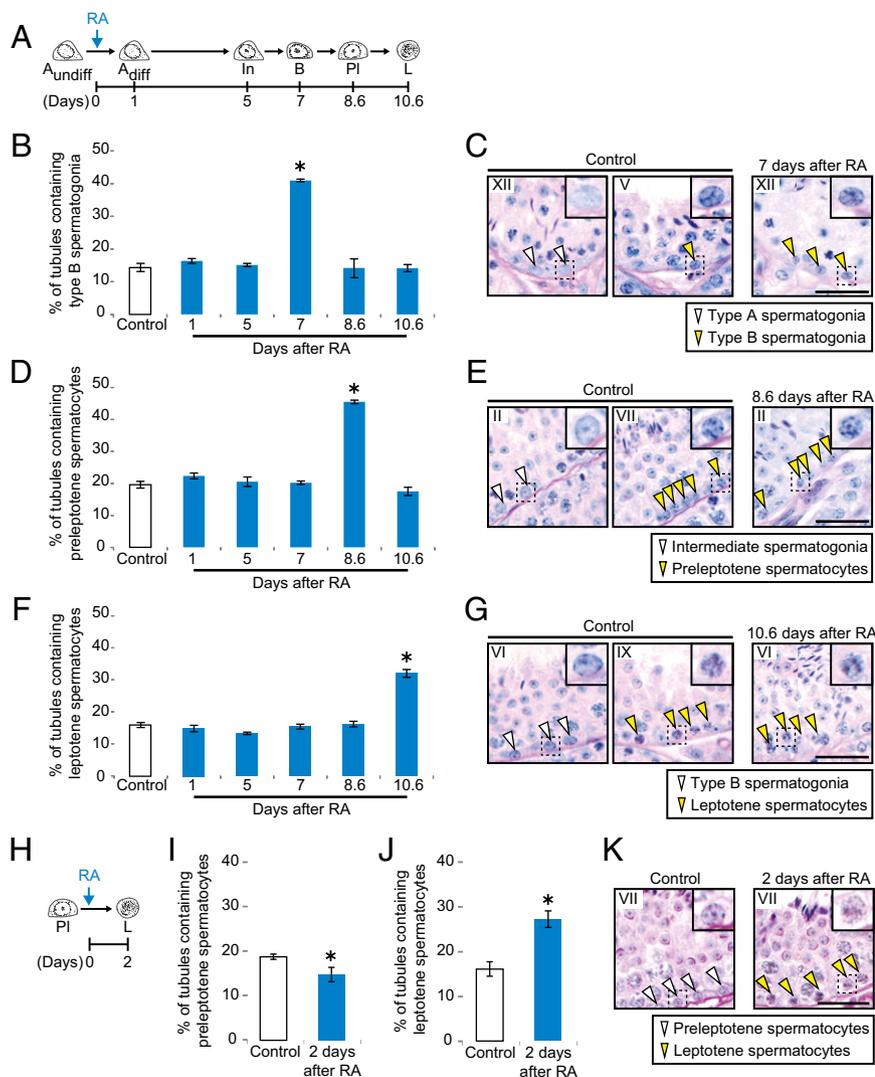


Fig. 5. Injected RA induces precocious spermatogonial differentiation and meiotic initiation. (A) Diagram of predicted germ-cell development after RA-induced spermatogonial differentiation. (B, D, and F) Percentage of tubules containing type B spermatogonia (B), preleptotene spermatocytes (D), or leptotene 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

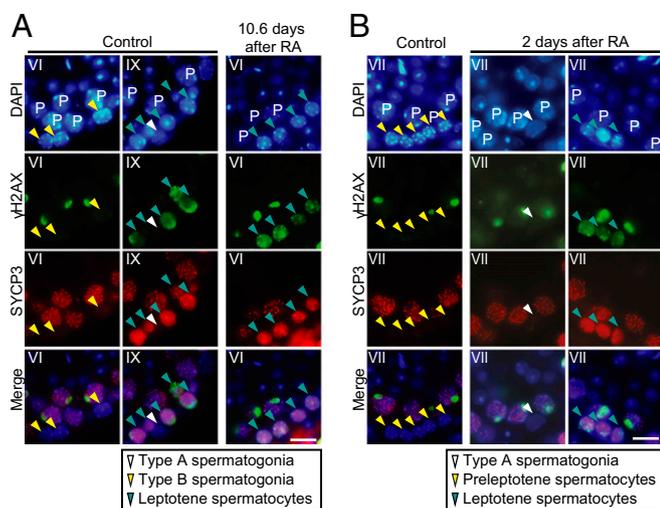


Fig. 6. Injected RA induces precocious expression of meiotic markers. (A and B) Immunostaining for γ H2AX (green) and SYCP3 (red), with DAPI counterstain (blue), on control and RA-injected testis cross-sections. Roman numerals indicate stages. Arrowheads in A: type A spermatogonia (white), type B spermatogonia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

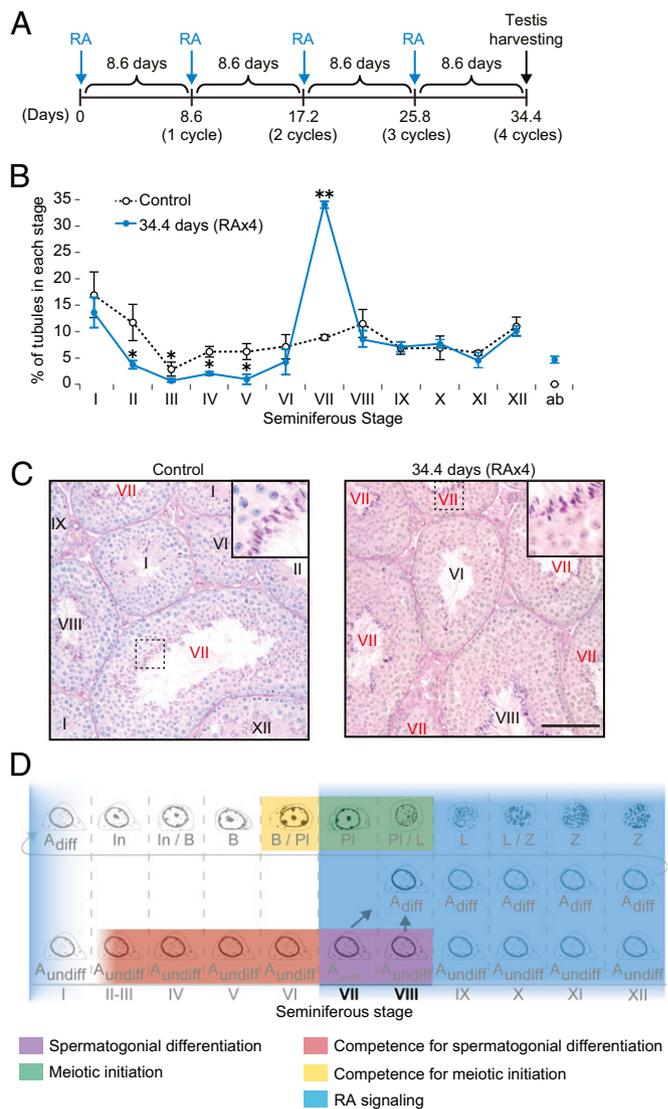


Fig. 7. Periodic RA–STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. (A) Diagram of four successive RA injections. Testes were harvested 34.4 d after the first RA injection. (B) Percentage of tubules in each stage of seminiferous cycle, in controls and after 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), *Stras8*-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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In-vitro differentiation of early pig spermatogenic cells to haploid germ cells

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ABSTRACT: Spermatogonial stem cells (SSCs) self-renew and contribute genetic information to the next generation. Pig is widely used as a model animal for understanding reproduction mechanisms of human being. Inducing directional differentiation of porcine SSCs may be an important strategy in exploring the mechanisms of spermatogenesis and developing better treatment methods for male infertility. Here, we established an *in-vitro* culture model for porcine small seminiferous tubule segments, to induce SSCs to differentiate into single-tail haploid spermatozoa. The culture model subsequently enabled spermatozoa to express the sperm-specific protein acrosin and oocytes to develop to blastocyst stage after round spermatid injection. The addition of retinoic acid (RA) to the differentiation media promoted the efficiency of haploid differentiation. RT-PCR analysis indicated that RA stimulated the expression of *Stra8* but reduced the expression of *NANOS2* in spermatogonia. Genes involved in post-meiotic development, transition protein 1 (*Tnp1*) and protamine 1 (*Prm1*) were upregulated in the presence of RA. The addition of an RA receptor (RAR) inhibitor, BMS439, showed that RA enhanced the expression of cAMP responsive-element binding protein through RAR and promoted the formation of round spermatids. We established an efficient culture system for *in-vitro* differentiation of pig SSCs. Our study represents a model for human testis disease and toxicology screening. Molecular regulators of SSC differentiation revealed in this study might provide a therapeutic strategy for male infertility.

Key words: retinoic acid / CREB / spermatid / post-meiotic / piglet / *in-vitro* spermatogenesis

Introduction

Mammalian spermatogonia originate from primordial germ cells. In rodents, type A_{single} spermatogonia (As) undergo self-renewal and proliferate into type A_{paired} spermatogonia (Ap) to initiate the

process of spermatogenesis. These spermatogonia subsequently form type A_{aligned} spermatogonia (Aal) and finally turn into type A1 spermatogonia without mitosis (Neuhaus *et al.*, 2017). These type As, Ap and Aal spermatogonia are collectively called undifferentiated spermatogonia. Some genes involved in the self-replication of

spermatogonial stem cells (SSCs), such as *NANOS2*, are essential to ensure a stable number of stem cells. *NANOS2* is a conserved zinc-finger RNA-binding protein that maintains the self-replication of A_s and A_{pr} spermatogonia (Sada et al., 2009). Its continuous expression is regulated by glial cell-derived neurotrophic factor (GDNF) through the GDNF family receptor alpha 1 (GFR α 1) on SSCs (Sada et al., 2012; Lee et al., 2013a). Type A1 spermatogonia then undergo mitosis and give rise to a series of differentiating spermatogonia types (A2, A3, A4, intermediate (In) and B type) before initiating meiosis as preleptotene primary spermatocytes. These differentiated type A1-derived spermatogonia express *Kit* and mitotic genes, which are specifically expressed before meiosis and stimulated by retinoic acid (RA) gene 8 (*Stra8*) (Shirakawa et al., 2013). The last phase of spermatogenesis is spermiogenesis, in which round haploid spermatids develop into mature flagellated spermatozoa. Spermatogenesis involves complex stages of cell differentiation and requires the involvement of various key factors, such as Sertoli cells, essential nutrients (amino acids and vitamins) and reproductive hormones (testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), as well as synergism between cytokines (Anjamrooz et al., 2006; Bialas et al., 2010) and construction of the required gene regulation network in spermatogenic cells (Manku and Culty, 2015). Several culture systems have been developed to investigate the complete process of spermatogenesis *in vitro* (Izadyar et al., 2003; Sato et al., 2011). However, owing to limited differentiation efficiency, these *in-vitro* models are not ideal for the practical production of functional sperm (Tesarik 2004 and Mahmoud, 2012).

Previous research reported that RA at a concentration of 10⁻⁸ M was sufficient for activating *Stra8* and promoting the onset of meiosis (Anderson et al., 2008; Zhang et al., 2011). RA is a metabolite derived from vitamin A (Busada and Geyer, 2016). When bound to its high-affinity RA receptor (RAR), RA affects the RA response elements in promoters of target genes to regulate transcription. The RAR includes three isomers, RAR α , RAR β and RAR γ . In newborn, pubertal and adult mammalian testes, RAR α is mainly located in testicular Sertoli cells. RAR γ is mainly expressed in differentiated spermatogonia. RA regulates spermatogonia differentiation mainly through RAR (Mark et al., 2015).

RA deficiency leads to elevated SSC numbers in the neonatal mouse testis (Agrimson et al., 2017). The differentiation of spermatogonia requires RA (Hogarth et al., 2015a). In mice, long-term vitamin A deficiency or RA antagonist (such as WIN18446) administration will block spermatogenesis at the early undifferentiated (Aal) stage and result in azoospermia and infertility. Replenishment of vitamin A or RA can restore fertility by inducing spermatogonial maturation from type Aal to type A1 (Paik et al., 2014). In Sertoli cells, RA enhances the expression of *Kit* ligand (the *Kit* receptor) and bone morphogenetic protein 4 (BMP4), which inhibits the expression of GDNF (Pellegriani et al., 2008). In undifferentiated spermatogonia, RA combines with RAR γ to stimulate the expression of *Kit* (Zhou et al., 2008) and *Stra8* genes (Busada et al., 2015). Recent studies have examined the effect of RA on inducing the differentiation of cultured SSCs *in vitro* (Dong et al., 2010).

After *in-vitro* differentiation, genetically modified SSCs could possibly be used for transgenic animal production and for treating genetic diseases. Induction of spermatogenesis *in vitro* might also contribute

to a therapeutic strategy for male infertility (Tanaka et al., 2018). Additionally, these *in-vitro* culture systems could be used for toxicology screening and for screening metabolic and nutritional conditions for the optimization of sperm production (Gadella and Ferraz, 2015). The miniature pig is a good animal model for understanding specific aspects of human reproduction, with advantages including similarities between mini-pig and human anatomy, physiology and pathology, with the benefit of short estrous cycles and a large number of piglets (Wang et al., 2016a). Less progress has been achieved on induced differentiation of pig SSCs compared with other livestock (Awang-Junaidi and Honaramooz, 2018). Fluorescent marker-labeled pig SSCs have been transplanted into a recipient testis, and 4 weeks later the fluorescence signal could be found in most of the testes (Honaramooz et al., 2002). In addition, SSCs of the Bama mini-pig have been shown to undergo *in-vitro* differentiation and develop to a post-meiotic stage in α -MEM supplemented with knockout serum replacement (KSR) and various induction factors (Zhao et al., 2018). This study will build on this foundation for accomplishing porcine spermatogenesis from SSCs *in vitro* and ultimately contribute to a better understanding of the mechanism of RA action during the initiation of meiosis and sperm formation.

Materials and Methods

Ethics approval

Piglet surgical biopsy was performed at the experimental station of the China Agricultural University and carried out in strict accordance with the protocol approved by the Animal Welfare Committee of the China Agricultural University.

Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich chemical company (St Louis, MO, USA) unless otherwise stated.

Isolation of piglet seminiferous tubule fragments

Testis tissue was obtained from five 2-month-old Chinese experimental miniature pigs. Testes were transported to the laboratory in phosphate buffered saline (PBS) supplemented with 100 mg/ml streptomycin (S1277) and 100 IU/ml penicillin (P7794). Under sterile conditions, albuginea was removed from testis. Seminiferous tubules were carefully disintegrated with fine-tipped tweezers and fully shredded. After decapsulation, seminiferous tubule fragments were dissociated by modified enzymatic digestion (Lee et al., 2013b; Zhang et al., 2017). Briefly, the seminiferous tubules were incubated with an enzyme cocktail containing 0.1 mg/ml collagenase type IV (C5138) and 1.0 μ g/ml DNase1 (DN25) at 37°C for 15 min, followed by neutralization with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The suspension was filtered with a 40-mesh filter, and single-cell and seminiferous tubule fragments were obtained. The single-cell and seminiferous tubule fragments were cultured in medium containing DMEM/F12 (Gibco, 10565-018), 20% KSR (Gibco, 10828010), 2 mmol/l L-glutamine (G8540), 1% non-essential amino acids (M7145), 10 ng/ml fibroblast growth factor (FE002),

Table I Primer sequences.

Gene (Accession NO.)	Primer sequence	Product size (bp)
NANOS2 (XM003127232.2)	5' GCAAACACAATGGGGAATCTC 3' 5' ACTGCGCGATAGAGAGACTG 3'	185
UCHL1 (AY459532.1)	5' AGTGCTGACAAGGCTCGGG 3' 5' CCGTGAGGGGAAAGAGCAG 3'	125
PLZF (XM021062868.1)	5' CCACCTCGTTTGGTCTTTCAG 3' 5' CCTTGTCCTCCATCCCAG 3'	252
c-kit (NM001044525.1)	5' CGCAGCGGGTATGATGTGTAT 3' 5' AAGGAAGTTGCGTTGGGTCTAT 3'	140
Stra8 (NM001285970.1)	5' GGTGCTTGAGCCTCGGGT 3' 5' TTGAGGCTGTGAGGTCAGAGTG 3'	122
CYP26B1 (XM003125033.5)	5' GCTTCCATTACCTCCCTTCG 3' 5' CAGGGTGATGCGTGGGAA 3'	146
RAR γ (U82629.1)	5' TGGGCAAATACACCACGAACT 3' 5' ATCAGGATGTCCAGGCAGGC 3'	196
Tnp1 (XM003133647.4)	5' AAGAGAGGTGGCAGCAAAAG 3' 5' TCACAAGTGGGAGCGGTAAT 3'	96
Cdyl (XM021100128.1)	5' ACTCGGAGCGTCCATACTGC 3' 5' CTGTCCGAAGGTTGTGAGGGT 3'	88
Prm1 (NM214253.1)	5' GTTGCCGACCCATAGC 3' 5' GCAGCACACGCTCTCC 3'	101
Hdac1 (XM013999116.2)	5' CTCAGGGCACCAAGAGGAAA 3' 5' GCCTTGTGAGGGCGATAGAT 3'	166
Bcl2 (XM021099593.1)	5' ACTTCTCTCGCTACCGC 3' 5' CACCCATCCCTGAAGAGC 3'	119
β -actin (DQ845171.1)	5' AGGTCATCACCATCGGCAA 3' 5' GAGGTCCTTGCGGATGTGC 3'	143

20 ng/ml GFR α 1 (SRP6474), 10 ng/ml GDNF (R&D Systems, Minneapolis, MN, USA, 512-GF), incubated at 37°C in 5% CO₂/air for 3 days.

In-vitro differentiation of SSCs

The seminiferous tubule fragments were suspended in medium (M) containing DMEM/F12, 5% KSR, 1% non-essential amino acids (M7145), 10 ng/ml stem cell factor (Peprotech, Rocky Hill, NJ, USA, 300-07), 10 ng/ml fibroblast growth factor (Peprotech, 100-18B), 25 ng/ml epidermal growth factor (Peprotech, 100-47), 10 ng/ml insulin–transferrin–selenium (Gibco, 41400-045), 2 mM L-glutamine (G3126), 0.05 IU/ml FSH (Merck Serono International SA, Geneva, Switzerland, 869001), 0.05 IU/ml LH (Merck, 869003), 0.1 μ mol/l testosterone (T6147) and 1% penicillin–streptomycin (Gibco, 15140) (Staub *et al.* 2000; Vigier *et al.*, 2004). The temperature was then maintained at 34°C in 5% CO₂/air. On the fifth day of culture, 1 mol/l RA (R2625) was added to one group, and after 48 h of incubation the medium was replaced by normal medium for further culture. One group of media was then supplemented with 1 mol/l RA for 96 h. To test for RA-specific actions, we added 5 μ M BMS493 (B6688), a pan-RAR

antagonist (Wang *et al.*, 2016b). In each culture system, half the medium was changed every 2 days. Cell growth was observed morphologically.

Quantitative real-time PCR

Cells from *in-vitro* culture Days 0, 3, 5, 7 and 9 and adult testis were collected to determine gene expression levels. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR was performed using a cDNA synthesis kit (Promega, Madison, WI, USA) and 2 μ g of total RNA according to the manufacturer's protocol. The SSC-related genes (GFR α 1, ubiquitin C-terminal hydrolase L-1 (UCHL1), promyelocytic leukaemia zinc finger (PLZF) and NANOS2 (Shi *et al.*, 2015)), SSC differentiation-related genes (Stra8, c-kit, RAR γ and cytochrome P450 family 26 enzymes B1 (CYP26B1)), an anti-apoptosis gene (Bcl2), genes with post-meiotic expression (transition protein 1 (Tnp1) and protamine 1 (Prm1)) and histone acetylation-related genes (chromodomain Y-like (Cdyl)) and histone deacetylase 1 (Hdac1) were detected by RT-PCR. β -actin was used as an internal control. The primer sequences are listed in Table I. Real-time PCR reactions were carried out with a Real Master Mix SYBR Green Kit (Tiangen, Corp, Beijing,

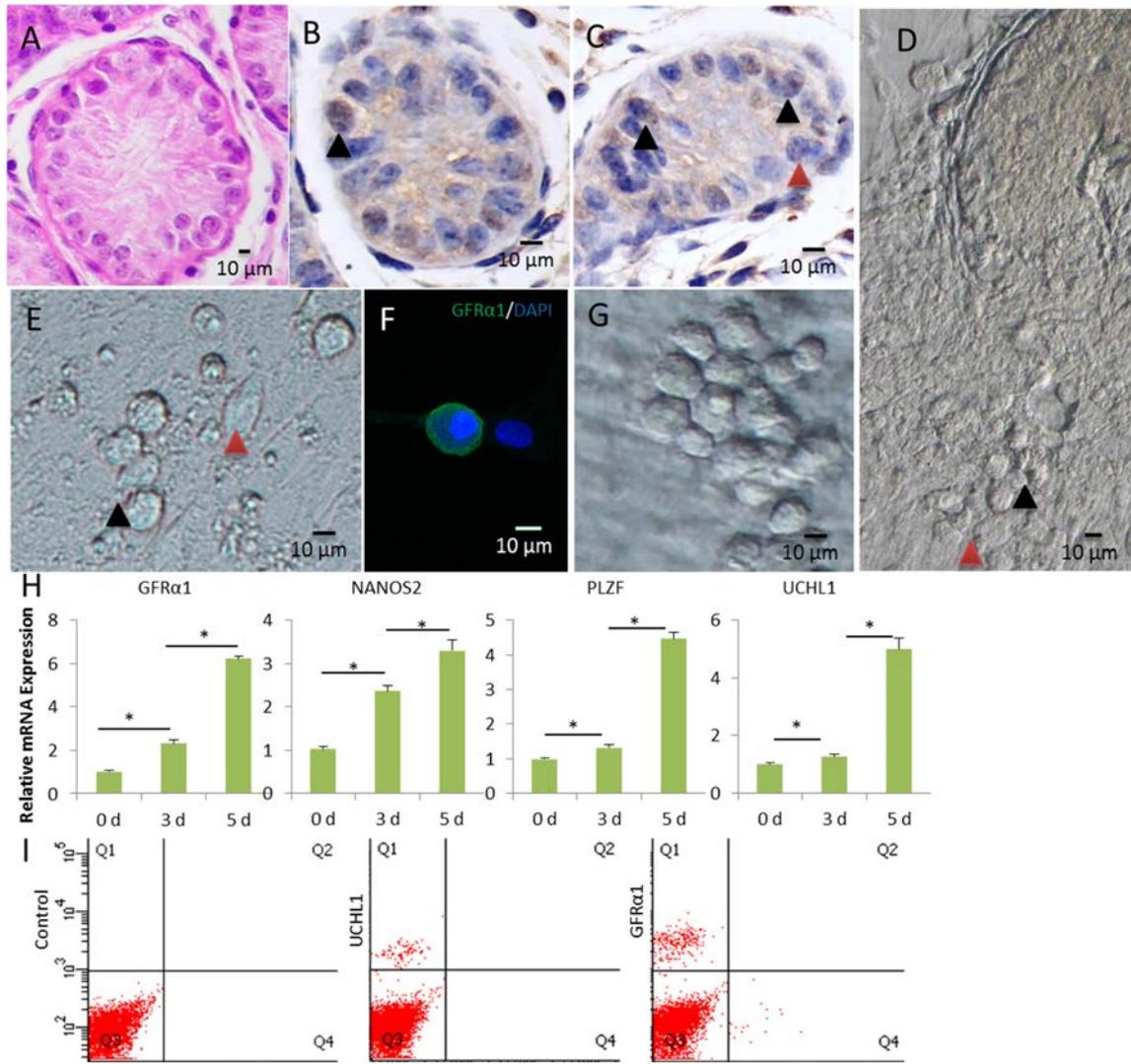


Figure 1 Male SSC culture. (A) H&E staining of the 2-month-old pig testis. (B) Immunohistochemical analysis of UCHL1 expression in the 2-month-old pig testis. SSC is indicated by black arrows. (C) The expression of RAR α in the seminiferous tubules of the porcine testis. Primordial germ cells are indicated by black arrows and Sertoli cells by red arrows. (D) *In-vitro* culture of porcine seminiferous tubules for 3 days. (E) Bridge and chain connections between SSCs in *in-vitro* culture for 3 days. Adherent Sertoli cells are indicated by red arrows; spermatogenic cells and bridges are indicated by black arrows. (F) Immunofluorescent analysis SSCs in *in-vitro* culture for 3 days: GFR α 1 (green) and DAPI-stained nuclei (blue). (G) SSC colony observation on Day 5. (H) Real-time PCR analysis of *GFR α 1*, *NANOS2*, *PLZF* and *UCHL1* mRNA levels in the *in-vitro* system on Days 3 and 5. Data are expressed as mean \pm SEM; * P < 0.05. (I) Flow cytometric analysis of UCHL1 and GFR α 1 on Day 5 of incubation.

China) using a Stratagene Mx300p (Agilent Technologies Inc., Santa Clara, CA, USA). Fold change of gene expression was calculated using the $2^{-\Delta\Delta ct}$ method and was expressed as a ratio of expression levels of treated groups to the expression level of the control group.

Flow cytometric analysis

The DNA content of cells was examined by flow cytometry. *In-vitro* cell suspensions adjusted to 1×10^6 cells/ml were collected at 9 days, and sperm from a mature pig was used as a control. The cells and sperm were fixed in 70% ethanol for 4 hr. After three washes in PBS, the cells were incubated at 37°C for 10 min in PBS plus 200 μ g/ml RNase I and

20 μ g/ml propidium iodide. Cells cultured for 5 days were examined by flow cytometry for germ cells. Briefly, the cells were fixed in 70% alcohol for 2 h, then washed twice in PBS and then re-suspended in PBS with bovine serum albumin (BSA) for 1 hr. The cells were then incubated with anti-UCHL1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-25800, diluted 1:200) and GFR α 1 (Santa Cruz Biotechnology, sc-6157, diluted 1:200) for 1 h. Cells were then washed three times in PBS by centrifugation at 500 \times g for 5 min, and then the secondary antibody was added and incubated for 45 min. Nonspecific binding of antibodies was eliminated by isotype control. The cells were then washed three times with PBS and re-suspended in 0.5 ml PBS for analysis by flow cytometry.

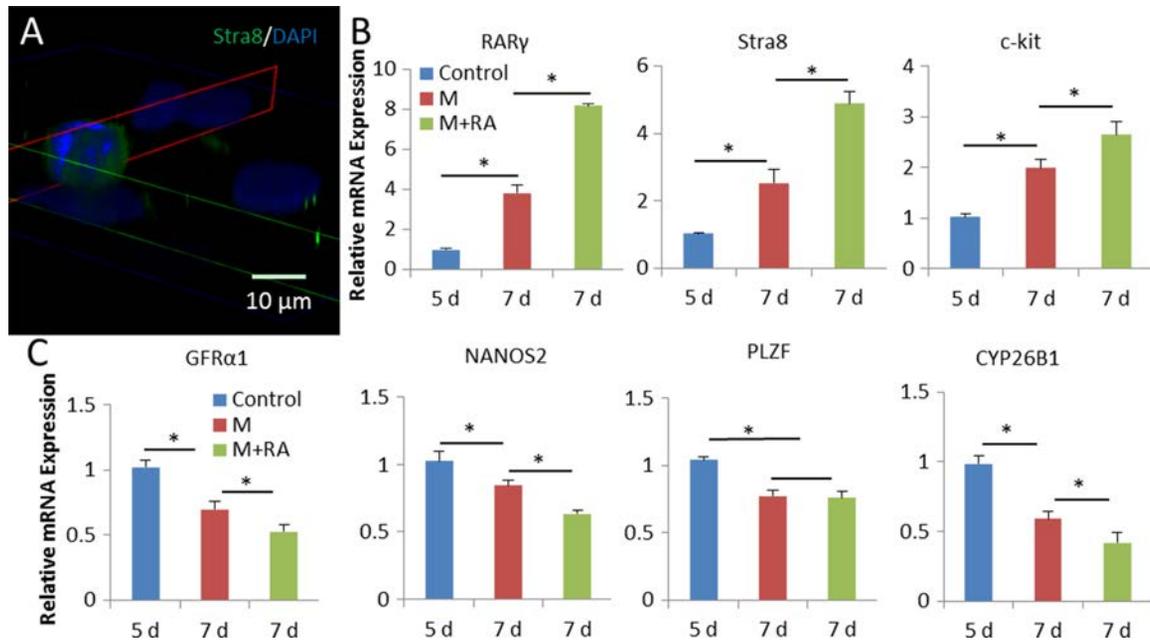


Figure 2 RA upregulated the expression of *Stra8* in the porcine SSC *in-vitro* differentiation system. (A) Immunofluorescent analysis: *Stra8* (green) and DAPI-stained nuclei (blue). (B) and (C) real-time PCR analysis of *RAR γ* , *Stra8*, *c-kit*, *GFR α 1*, *NANOS2*, *PLZF* and *CYP26B1* mRNA levels in the *in-vitro* system at various times (Days 5 and 7). The control is the group without induction (SSCs on Day 5 of incubation), M is the group that was induced to differentiate with basic medium, and M + RA is the group with RA treatment. Data are expressed as mean \pm SEM; * P < 0.05.

Immunofluorescence analysis and ELISA

Cells were examined by immunofluorescence staining after 3 days of culture for *GFR α 1*, a marker for SSCs (Luo *et al.*, 2006); after 7 days of culture for *Stra8* (Abcam Inc., Cambridge, MA, USA, ab49602), a marker for differentiated spermatogonia; and after 9 days of culture for acrosin (Bioss, Beijing, China, bs-5151R), a marker for spermatozoa (Deng *et al.*, 2017a). Briefly, cells were fixed in 70% alcohol for 2 h and then washed twice in PBS. Slides were blocked with 1% BSA for 1 h at room temperature, and primary antibody (diluted 1:200) was added to the solution and incubated for 4 h. Slides were rinsed twice and washed three times with PBS for 5 min. Secondary antibody (1:500) was incubated for 1 h at room temperature, followed by the same PBS washes, and nuclei were stained with DAPI.

Cells cultured for 9 days were collected for cAMP responsive element binding protein (CREB) detection by an ELISA kit (Hermes Criterion Biotechnology, Vancouver, Canada) following the manufacturer's protocol.

Immunohistochemistry

Testis samples from 2-month-old pigs were fixed with 4% paraformaldehyde. The samples were cryo-embedded in OCT compound and then cut into 7 μ m thick sections and stained using hematoxylin and eosin (H&E) for histological analysis of the seminiferous tubules. The *UCHL1* (Almunia *et al.*, 2018) and *RAR α* (Channabasappa *et al.*, 2014) (Santa Cruz Biotechnology, sc-551) expression patterns were examined by immunohistochemistry. Briefly, after washing three times with PBS, the slides were incubated in PBS containing 1% BSA

for 1 h at room temperature. Primary antibodies (diluted 1:200) were added to the solution. After 4 h of incubation, the secondary antibody was applied for 1 h. Staining was visualized using a DAB substrate kit.

Intracytoplasmic microinjections

Ovaries were collected from a slaughterhouse. Cumulus cells and cumulus-oocyte complexes were selected and cultured in *in-vitro* maturation medium that included 10% FBS TCM-199 (Gibco), 10 ng/ml epidermal growth factor, 10% porcine follicular fluid, 10 IU/ml equine chorionic gonadotrophin, 5 IU/ml human chorionic gonadotrophin and 2 mM glutamine. Oocytes extruding the first polar body were selected for injection of round spermatids collected from testes (Ogonuki *et al.*, 2006). Spermatids <10 μ m in diameter with single flagella were collected from the *in-vitro* system and used for microinjection (Sousa *et al.*, 1998). Micromanipulation was performed in TCM-199 medium supplemented with 3 mg/ml BSA (A6003) and 0.5 mM HEPES. Oocytes were activated with 5 μ M ionomycin for 5 min before injection (Rho *et al.*, 1998; Nakai *et al.*, 2016). Cell membranes of spermatids were disrupted by repeated blowing with an injection needle, and then spermatids were injected into the cytoplasm of oocytes. Intracytoplasmic injection was finished within 1 h after activation. Recovered couplets were transferred into development medium, porcine zygote medium (PZM-3), for recovery at 38°C and 5% CO₂ for 30 min, and then activated with 10 μ g/ml cycloheximide (C1988) for 4 h (Liu *et al.*, 1998, 2015). After activation, reconstructed embryos were cultured at 38°C in 5% CO₂ for development, and the development of double pronuclei in reconstructed embryos

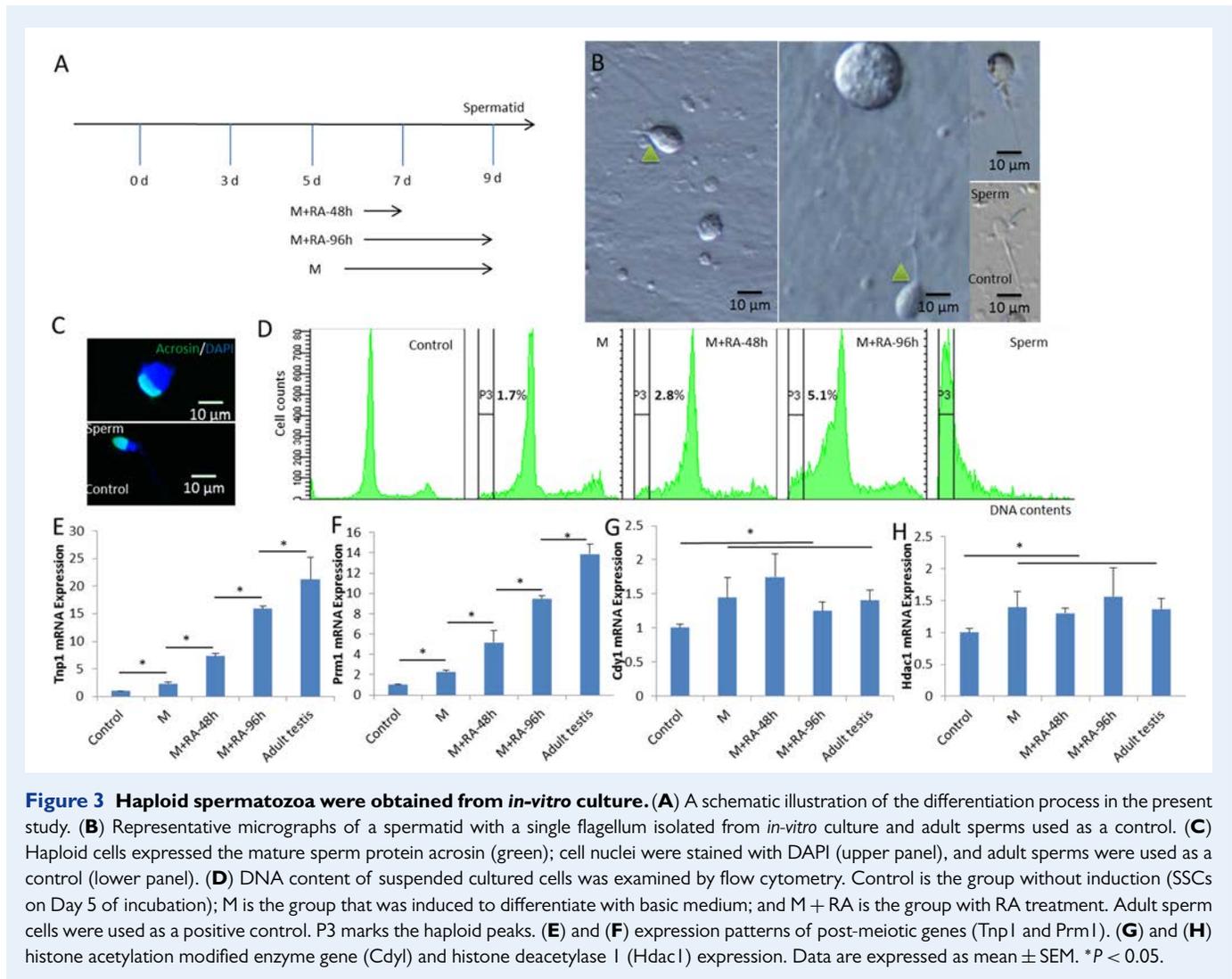


Table II Ratio of haploid spermatozoa in suspended cells 9 days after SSC differentiation.

In-vitro differentiation medium	Haploid ratio (%)
M	1.7 \pm 0.32 ^c
M+RA-48h	2.8 \pm 0.55 ^b
M+RA-96h	5.1 \pm 0.83 ^a

Note: ^{a, b, c} Difference among M, M+RA-48h and M+RA-96h ($P < 0.05$).

was observed by orcein staining. After 12 h, reconstructed embryos were collected and rinsed twice with PBS and then embryos fixed with acetic acid and ethanol (V/V = 1:3) for 48 h. The embryos were drained of the fixative and placed in 1% orcein solution for 18 h, then washed twice with glycerol, glacial acetic acid and water (V/V/V = 1:1:3). A microscope was used to observed pronucleus (Casillas et al., 2015; Wei et al., 2016). Blastocysts were observed on Day 7.

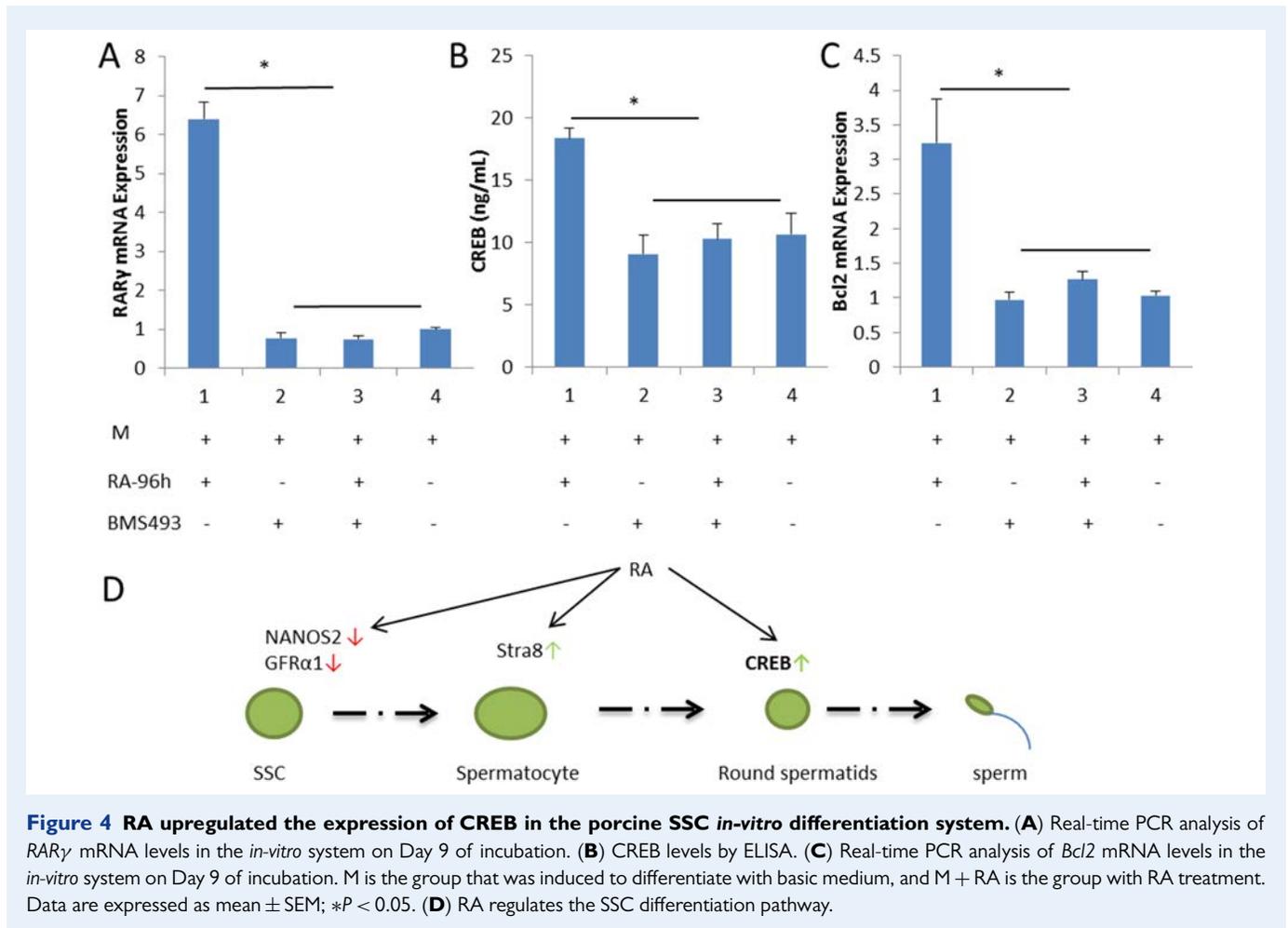
Statistical analyses

All experiments were repeated at least three times (with cells from three different testes). One-way ANOVA was used to determine statistical significance with the Duncan's test used to determine the statistical significance between the relative groups. Statistical analysis was conducted using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All data were expressed as mean \pm SEM. Differences were considered to be significant when $P < 0.05$.

Results

Identification of pig SSCs

The H&E-stained sections of 2-month-old porcine testis revealed that only undifferentiated spermatogonia were present in seminiferous tubules (Fig. 1A). Expression of UCHL1 was detected in porcine SSCs by immunostaining (Fig. 1B). Both Sertoli cells and spermatogenic cells expressed RAR α (Fig. 1C). Adherent Sertoli cells had grown out from small seminiferous tubule segments when observed 3 days after plating. At this stage, some spermatogenic cells gathered around the Sertoli



cells, free but close to the surface of Sertoli cells (Fig. 1D). Thereafter, bridge and chain connections between cells were observed (Fig. 1E), and these cells expressed GFRα I (Fig. 1F). On Day 5 of RA treatment, SSC colonies were observed (Fig. 1G). Expression levels of GFRα I, UCHL1, PLZF and NANOS2 transcripts were significantly higher on Day 5 compared with Day 3 of incubation (Fig. 1H), and simultaneous flow cytometric analysis identified UCHL1 and GFRα I protein expression in the culture system (Fig. 1I).

RA upregulated the expression of STRA8 in porcine SSCs in vitro

After 48 h induction of SSCs on Day 5, the expression of Stra8 was localized to spermatogenic cells (Fig. 2A). Expression of RARγ mRNA levels was significantly elevated in the RA group compared with the M group (P < 0.05). The expression of gene Stra8 and c-kit was also significantly higher in the RA group than that in M group (P < 0.05), indicating that RA may promote the expression of Stra8 and c-kit through its receptor (Fig. 2B). Expression of NANOS2 and GFRα I mRNA levels was reduced in the RA group compared to that before induction (P < 0.05). Reduced expression of PLZF mRNA was also found in the RA-treated relative to the M group (P < 0.05); however, there was no significant difference compared with the control group

(Fig. 2C). Additionally, decreased expression of CYP26B1 mRNA was observed in the RA group compared with the M and control groups. These results suggest that RA reduced the expression of NANOS2 and GFRα I in spermatogonial cells and promoted the expression of Stra8 in meiotic spermatogenic cells.

In-vitro differentiation of SSCs into sperm-like cells

The differentiation of a single tail to Sa spermatid or Sd type spermatozoa (Fig. 3A and B) was observed on Day 9 of incubation. Immunofluorescence showed some cells in the culture system expressed round spermatid-specific acrosin (Fig. 3C). Ploidy analysis revealed that haploid efficiency of the M + RA-96 h group was 5.1 ± 0.83%, higher than that of the M + RA-48 h group and M group (Fig. 3D and Table II). At the later stage of meiosis, Tnp1 and Prm1 were expressed at significantly higher levels in the M + RA-96 h group than in the other groups (Fig. 3E and F). There was no significant difference in histone acetylation modifying enzyme Cdy1 and Hdac1 (histone deacetylase gene expression within the groups (Fig. 3G and H). The above results indicate that in the in-vitro induction culture system of spermatogenic cells, continuous RA treatment can significantly increase the differentiation rate of haploid cell and sperm formation in vitro.

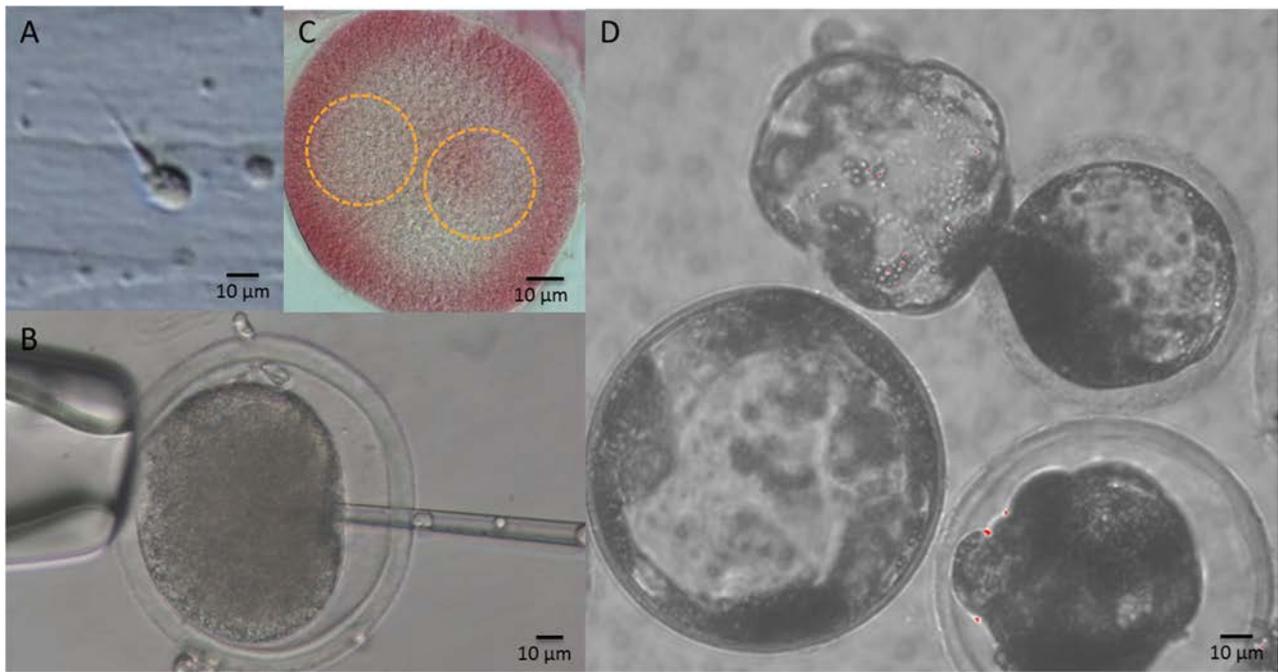


Figure 5 Haploid spermatozoa obtained from *in-vitro* differentiation. (A) Single-tail spermatid obtained from *in-vitro* differentiation. (B) Spermatid intracytoplasmic injection into an oocyte. (C) Nuclear reconstructed embryos. (D) Reconstructed embryos developed to the blastocyst stage.

RA upregulated the expression of CREB in porcine SSCs *in vitro*

The expression of *RARγ* mRNA was significantly lower in the RAR inhibitor BMS493 group than in the M + RA-96 h group after 9 days of induction. The content of CREB protein was significantly higher in the M + RA-96 h group than in other groups, and the content of CREB in the RAR inhibitor BMS493 group was lower than that of M + RA-96 h group (Fig. 4A and B). The addition of RA promoted the expression of anti-apoptotic Bcl2 mRNA, but the addition of BMS493 inhibited this elevation (Fig. 4C). These results suggest that RA promotes the post-meiotic germ cell expression of CREB through its specific receptors (Fig. 4D).

The cultured porcine haploid spermatozoa exhibit developmental potential

Cultured pig round spermatids were injected into metaphase II-stage oocytes (Fig. 5A and B). Injected oocytes formed double-pronuclear reconstructed embryos, as shown by orcein staining (Fig. 5C) and further developed to cleavage and blastocyst stages (Fig. 5D). The rate of blastocyst ($14.62 \pm 3.12\%$) was significantly lower than that of the single sperm injection group ($24.60 \pm 2.75\%$) ($P < 0.05$) but not significantly different to that of the *in-vivo* round spermatid group (16.36 ± 2.25) (Table III). These findings indicate that the culture-derived pig spermatid with a single flagellum had developmental potential *in vitro*.

Discussion

In this study, porcine SSCs were successfully induced to differentiate into haploid spermatozoa *in vitro*. After 9 days of culture, spermatids with a single flagellum were formed (Gerton and Millette, 1984), indicating that a much shorter time span was required *in-vitro* compared with *in-vivo* spermatogenesis (Weiss et al., 1997). By adding RA, the differentiation efficiency of haploid cells was enhanced. The RA found in testes is mainly derived from intratesticular synthesis, and testes express a variety of transporters and enzymes related to the synthesis and metabolism of RA. RA can be degraded by CYP26B1, which localizes to perivascular myocyte-like cells and regulates RA expression levels in the seminiferous epithelium (Hogarth et al., 2015b). Disruption of key enzyme genes in RA synthesis, such as *Rdh10* or *Aldh1a1-3*, leads to RA-deficient mouse testes with spermatogenic arrest at the stage of undifferentiated spermatogonia (Raverdeau et al., 2012; Tong et al., 2013). RA regulates spermatogonial differentiation, spermatocyte meiosis and later stages of spermatogenesis (Endo et al., 2017). In addition, RA has been shown to induce undifferentiated spermatogonial cells to form differentiated spermatogonial cells *in vitro* (Nolte et al., 2010; Yang et al., 2016). RA triggers spermatogonial differentiation via direct or indirect downregulation of the zinc finger PLZF protein (Dann et al., 2008), which maintains SSCs in an undifferentiated state. In addition, RA directly activates the phosphorylation of Kit, which regulates the synthesis of DNA in mitotic spermatogonia and the initiation of meiosis via MAPK and PI3K signaling pathways (Dolci et al., 2001). In mice lacking the RA target gene *Stra8*, undifferentiated spermatogonia accumulate in high numbers as early as

Table III Embryo development of pig oocytes after intracytoplasmic spermatozoa injection (ICSI) or round spermatid injection (ROSI).

	MII-oocytes	IVC	2-cell (%)	Morulae (%)
ROSI (in-vitro cultured)	150	130	52 (40.00±5.25) ^b	19 (14.62±3.12) ^b
ICSI (in-vivo separated)	135	126	80 (63.49±4.40) ^a	31 (24.60±2.75) ^a
ROSI (in-vivo separated)	135	110	63 (57.27±3.82) ^a	18 (16.36±2.25) ^b

Note: Different superscript letters indicate significantly different values ($P < 0.05$).

10 days after birth, whereas differentiating spermatogonia are depleted (Endo *et al.*, 2015). The RNA-binding protein NANOS2 can silence genes involved in spermatogonial cell differentiation and meiotic entry, such as *Stra8*, and it is required to maintain the function and survival of undifferentiated spermatogonia (Tassinari *et al.*, 2015). The current results from the *in-vitro* differentiation of porcine SSCs showed that RA downregulated the expression of *NANOS2*, *GFR α 1*, *PLZF* and *CYP26B1* in spermatogonia but promoted the expression of *Stra8* in meiotic spermatocytes and promoted the initiation of meiosis.

The formation of mature sperm is associated with RA. In *RAR α* knockout mice, the first wave of spermatogenesis is blocked at step 8 spermatids but can be rescued by the specific overexpression of *RAR α* in round spermatids (Chung *et al.*, 2004). The *RAR* antagonist BMS-189453 blocks mouse spermatogenesis (Chung *et al.*, 2011). Cyclic AMP plays a role in the activation of post-meiotic genes, such as *Tnp* and *Prm*, and many genes involved in meiosis include CREB sequence (Deng *et al.*, 2016). CREB is an important transcription factor that is differentially regulated in various cell types. The cAMP responsive-element modulator (CREM), a member of CREB family, is an important transcriptional activator during spermatogenesis, especially in post-meiotic germ cells (Deng *et al.*, 2017b). Loss or deficiency of CREB and/or CREM can lead to a reduction of spermatogenesis and male infertility (Adham *et al.*, 2005). Inactivation of CREM in mice results in an increased rate of apoptosis in the round spermatid stage and the simultaneous expression of apoptosis-related genes (Kosir *et al.*, 2012). RA rapidly activates CREB without using RARs in normal human tracheobronchial epithelial cells. RA rapidly activates protein kinase C and transmits an activation signal to phosphorylate nuclear CREB via the Ras/ERK/Rsk pathway, thereby increasing its trans-activation activity (Hong *et al.*, 2008). Shan *et al.* (2008) found that active CREB protein was increased after treatment with 5 μ M RA during the differentiation/formation of the embryoid body. In the *in-vitro* induction culture system for porcine spermatogenic cells, RA significantly increased the differentiation rate of haploid germ cells. RA can promote the expression of CREB in post-meiotic spermatogenic cells and promote the rate of sperm formation *in vitro*. This study also revealed that elevated expression of CREB and upregulated expression of *Bcl2* was associated with decreased apoptosis of the cultured porcine reproductive cells *in vitro*.

Conclusions

In this study, we successfully used the *in-vitro* culture model of porcine small seminiferous tubule segments to induce SSCs to differentiate

into single-tail haploid spermatozoa with the potential for further development. When spermatogenic cells in the *in-vitro* culture system were treated with RA, the expression levels of *Stra8* and CREB were upregulated, likely enhancing the efficiency of haploid cell production. Through RAR, RA promoted CREB expression, which supports more efficient spermatid differentiation and sperm production. The *in-vitro* culture, which we setup in this study, might provide new insights into the *in-vitro* differentiation of human SSCs. We have also tried to explore the role and mechanism of RA in anaphase of meiosis, and we believe it could bring benefits to infertile patients.

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Authors' roles

S.-L.D., Z.-X.L. and Y.-X.L. conceived and designed the experiments. S.-L.D., W.-Q.J. and D.-P.H. performed the experiments. K.Y. analyzed the data. S.-T.W. and H.-Y.W. contributed reagents, materials or analysis tools. B.-L.Z., Y.Z. and K.Y. wrote the paper.

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Conflict of Interest

The authors declare that they have no competing interests.

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ARTICLE OPEN

The adult human testis transcriptional cell atlas

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Human adult spermatogenesis balances spermatogonial stem cell (SSC) self-renewal and differentiation, alongside complex germ cell-niche interactions, to ensure long-term fertility and faithful genome propagation. Here, we performed single-cell RNA sequencing of ~6500 testicular cells from young adults. We found five niche/somatic cell types (Leydig, myoid, Sertoli, endothelial, macrophage), and observed germline-niche interactions and key human-mouse differences. Spermatogenesis, including meiosis, was reconstructed computationally, revealing sequential coding, non-coding, and repeat-element transcriptional signatures. Interestingly, we identified five discrete transcriptional/developmental spermatogonial states, including a novel early SSC state, termed State 0. Epigenetic features and nascent transcription analyses suggested developmental plasticity within spermatogonial States. To understand the origin of State 0, we profiled testicular cells from infants, and identified distinct similarities between adult State 0 and infant SSCs. Overall, our datasets describe key transcriptional and epigenetic signatures of the normal adult human testis, and provide new insights into germ cell developmental transitions and plasticity.

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INTRODUCTION

Human spermatogenesis involves the differentiation of adult spermatogonial stem cells (SSCs) into mature sperm through a complex developmental process, regulated by the testis niche. Human SSCs must carefully balance their self-renewal and differentiation, and then undergo niche-guided transitions between multiple cell states and cellular processes—including a commitment to mitosis, meiosis, and the subsequent stages of sperm maturation, which are accompanied by chromatin repackaging and major morphological changes.^{1,2} Through a wide range of approaches, considerable progress in understanding gametogenesis and germline-niche communication has been achieved in mice.^{3,4} In contrast, in humans, although adult testis physiology is well described,^{5–7} much less is known about SSCs and their regulation. Ultimately, a full understanding will require the integration of molecular, genomic, proteomic and physiological approaches.

Toward this goal, single cell RNA-seq (scRNA-seq) approaches can effectively delineate cell types, uncover heterogeneity, and infer developmental trajectories.⁸ These approaches have recently been applied to human fetal germ cells, providing important new biological insights.⁹ Single-cell approaches are well suited for addressing fundamental questions about SSCs, differentiating spermatogonia and gametogenesis. For example, what are the main molecular features that enable SSCs to serve as the long-term adult germline stem cells? How do SSCs transition from their initial, most naïve and quiescent states to spermatogonia that will eventually commit to meiosis? Are these transitions irreversible, or

do spermatogonia possess bidirectional plasticity that helps ensure a lifelong pool of SSCs? Beyond spermatogonia, what are the subsequent sequential transcription and signaling programs that accompany gametogenesis? How are these processes influenced by communication with niche cells—what are the specific signaling and transcription pathways that regulate self-renewal, proliferation rates, metabolism, and transitions between differentiation states? Importantly, these questions overlap conceptually with other stem cell systems. Here, we aim to utilize single-cell transcriptome analysis from the full repertoire of germline and niche cells to address these questions.

Prior scRNA-seq efforts characterizing spermatogonia enriched via cell surface markers have provided initial insights into human spermatogenesis.¹⁰ However, thanks to new technological advances, it is now possible to use unbiased approaches to assess germline and niche cell transcriptional profiles. Here, we performed extensive scRNA-seq characterization of unselected human testicular cells of young adults using the 10× Genomics Chromium platform—yielding a transcriptional cell atlas of all cell types in the testis, including germline and niche cells. We delineate five distinct spermatogonial states in adults, including a novel early SSC state, termed State 0, which displays high similarity to infant SSCs. We further describe the genic and non-coding RNA expression programs that accompany spermatogenesis. Intriguingly, combining RNA ‘velocity’ analyses¹¹ with chromatin mapping and DNA methylation (DNAm), we provide computational and molecular evidence that human spermatogonia possess considerable transcriptional/state

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plasticity, suggesting a conceptual framework for human spermatogonial homeostasis, similar to that described in other stem cell systems.

RESULTS

Cell partitioning through the analysis of single cell transcriptomes We isolated single cells from whole-testis of 3 individuals using a standard two-step procedure of enzymatic digestion and physical filtering.^{7,10} For each donor, two separate technical replicates were performed (Fig. 1a), resulting in six datasets. From a total of ~7000 cells, 6,490 passed standard quality control (QC) dataset filters and were retained for downstream analysis. We obtained ~250 K reads/cell which enabled the analysis of ~2500 genes/cell. The sequencing saturation rate was >83%, and technical replicates were highly similar ($r > 0.96$; Supplementary information, Fig. S1a).

Cell partitioning via t-distributed stochastic neighbor embedding (tSNE) analyses¹² identified 13 clusters (Fig. 1b; Supplementary information, Table S1), with only minor variation based on batch/experiment or donor origin (Fig. 1c; Supplementary information, Fig. S1b and c). Cluster identity was assigned based on known cell-type marker expression (Fig. 1d; Supplementary information, Fig. S2). Clusters 9–13 correspond to macrophage, endothelial, myoid, Sertoli and Leydig cells, respectively (Fig. 1d; Supplementary information, Fig. S2a). Germline-specific markers were expressed solely in Clusters 1–8 (e.g., *DAZL* and *MAGEA4*; Fig. 1d; Supplementary information, Fig. S2b). Moreover, known SSC markers (e.g., *UTF1*, *ID4* and *FGFR3*), differentiating markers (e.g., *KIT* and *DMRT1*), meiosis markers (e.g., *SYCP3*, *SPO11*, and *MLH3*), spermatid structure proteins (e.g., *SPAG6*, *ZPBP*, *CAMK4* and *CREM*) and nuclear condensation/protamine repackaging factors (e.g., *TNP1* and *PRM2*) showed sequential expression peaks in Clusters 1 to 8, respectively—mirroring the temporal order of gametogenesis.

Human-mouse comparisons in intra-niche and niche-germline interaction

We began by describing the niche cell datasets (Fig. 2). Testicular macrophages (Cluster 9) promote spermatogonia maintenance,^{13,14} and were identified by multiple specific markers (i.e., *CD14*, *CD163*, *C1QA*; Fig. 1d and 2a). Previous work showed that mouse Sertoli cells help maintain CXCR4⁺ spermatogonia population by secreting CXCL12, the ligand for CXCR4.¹⁵ Interestingly, in humans, RNA encoding CXCL12 was observed in Leydig cells, while the *CXCR4* receptor was expressed in both macrophages and spermatogonia (Fig. 2a), suggesting that CXCL12-CXCR4 promotes co-localization of macrophages and spermatogonia in humans. Furthermore, *CSF1R*, the receptor for *CSF1*, was specifically expressed in macrophages, whereas in mice its expression is confined to spermatogonia.¹⁶

In endothelial cells (Cluster 10, marked by *VWF* and *PECAM1*), the receptor (*NOTCH4*) and the downstream signaling factors (*JAG1*, *HES1* and *MAML1*) for NOTCH signaling were specifically up-regulated (Fig. 2b). Hedgehog signaling is important for mouse fetal myoid and Leydig cell development;^{9,17} but the receptors (*PTCH1*, *PTCH2*), and downstream signaling components (*GLI* and *IGFBP6*) of the Hedgehog pathway were highly expressed in human adult myoid (Cluster 11, marked by *MYH11* and *ACTA2*) and Leydig cells (Fig. 2c), indicating that both Hedgehog and NOTCH signaling activity persists through adulthood in human testes. Sertoli cells (Cluster 12, marked by *SOX9* and *AMH*) express *ITGA6*, an integrin found in the basal membrane of seminiferous tubules in humans (Fig. 2d). Notably, Sertoli cells express *WFDC2*, which is known to encode an epididymis protein that may promote sperm maturation,¹⁸ and *PRND*, which encodes a glycosylphosphatidylinositol-anchored glycoprotein with a putative interaction role with receptors from germ cells.¹⁹

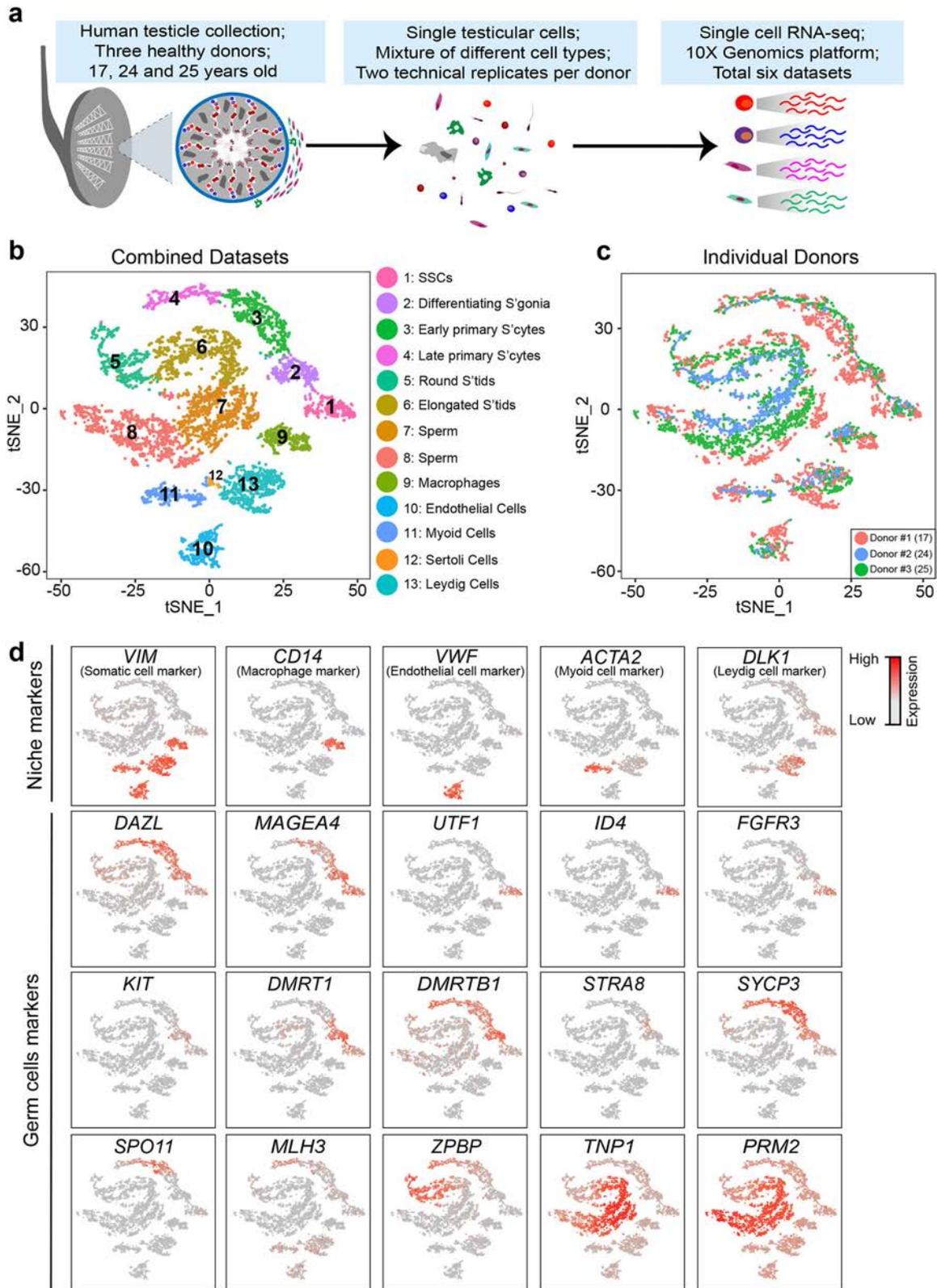
Leydig cells (Cluster 13, marked by *DLK1* and *IGF1*) also expressed specific genes (Fig. 2e), including those encoding IGF binding proteins (*IGFBP5* and *IGFBP3*) and INHBA, a subunit of both inhibin and activin, and the extracellular matrix protein *VIT*. Interestingly, key genes for testosterone biosynthesis, *STAR* and *HSD17B3*, were expressed in both Sertoli and Leydig cells, while the expression of the responsive genes, *SHBG* and *SRDS5A2*, were observed in maturing sperm (Fig. 2f). Retinoic acid (RA) induces germ cell differentiation, and enzymes for RA synthesis, *ALDH1A1* and *ALDH1A3*, were specifically expressed in Leydig and myoid cells; while *STRA8*, an RA target gene, was only observed during the transition of spermatogonia to spermatocytes (Fig. 2f). Interestingly, we found the WNT ligand, *WNT2B*, was expressed primarily in myoid cells, while *WNT2B* receptors were confined to primary spermatocytes, suggesting a role for *WNT2B* in human meiosis (Fig. 2f). *PDGFB* was expressed in endothelial cells, and its receptors *PDGFRA* and *PDGFRB* were found in Leydig and myoid cells, indicating that endothelial cells may indirectly affect germ cell development, via cross-talk mechanisms with other niche cells. Taken together, our data highlight both similarities and notable differences in germline-niche interactions in humans and mice that warrant further detailed functional investigations.

Pseudotime and clustering analyses reveal dynamic gene expression patterns during spermatogenesis

Noticeably, the germ cell clusters (1–8) formed a wave-like continuum, sometimes separated by distinct bottlenecks, that recapitulated the temporal order of spermatogenesis. Pseudotime analysis²⁰ provided an arrow vector which aligned with the developmental order of gametogenesis (Fig. 3a). Clustering analysis of genes (rows) while fixing the order of cells (columns) along pseudotime, revealed 12 distinct gene cohorts. Gene ontology (GO) analysis of these clusters yielded a dynamic developmental, cellular and metabolic sequence of events, consistent with well-organized germline development (Fig. 3b; Supplementary information, Table S2). Next, differential analysis (bimodal test; adjusted p -value < 0.01; $|\log_{2}FC| > 0.25$) identified differentially-expressed genes (Fig. 3c; Supplementary information, Table S3). As expected, ‘cell cycle’, ‘meiosis’ and ‘spermatogenesis’ were significantly-enriched GO terms during spermatogenic progression. Interestingly, >4600 genes were differentially expressed (2525 up and 2101 down) during the transition from spermatocytes to round spermatids, displaying the most dramatic transcriptomic change.

Germline expression dynamics of transposable elements, lncRNAs and XIST

Single-cell datasets allow a refined examination of transposable elements (TE) and long non-coding RNAs (lncRNAs) (Supplementary information, Figs. S3a–c and S4a–b). Pseudotime and clustering analyses identified dynamic TE and lncRNA programs during spermatogenesis. Notably, LTR12C/D/E and the active TEs SVA_D and AluYa5 showed high expression during early spermatogenic stages. By contrast, LTR10A and LTR40c expression peaks during late spermatogonial or post-meiotic stages; and satellite and multiple MLT-family TEs at spermatid and sperm stages (Supplementary information, Fig. S3d). Moreover, we explored X chromosome inactivation and meiotic sex chromosome inactivation (MSCI) during spermatogenesis.^{21,22} Notably, we observed *XIST* expression during spermatogonial stages (Supplementary information, Fig. S4c and d), and unexpectedly selective attenuation of genes that are near the X-inactivation center during spermatogonial stages (Supplementary information, Fig. S4e and f), suggesting a role for *XIST*-mediated silencing in this process. Overall, our datasets and analyses provide a comprehensive resource to study TEs and lncRNA expression dynamics during male germline development.



Analysis of meiotic cells reveals dynamic transcriptional programs and key factors during meiotic transition

Next, we singled out Clusters 3–4 (Fig. 1b) and performed re-clustering of the meiotic cells, which revealed five sub-clusters (Fig. 4a). Using known markers^{23,24} (Fig. 4b), we assigned pre-

leptotene, leptotene, zygotene/early pachytene, late pachytene, and diplotene cell types, consistent with the pseudotime developmental order. Secondary spermatocytes were under-represented consistent with their rapid progression into round spermatids.²⁵ Gene clustering analysis (Fig. 4c) identified five

Fig. 1 Single cell transcriptome profiling from healthy adult whole testes. **a** Schematic illustration of the experimental workflow. **b** tSNE and clustering analysis of combined single-cell transcriptome data from human testes ($n = 6490$). Each dot represents a single cell and is colored according to its cluster identity as indicated on the figure key. The 13 cluster identities were assigned based on marker gene expression shown in Fig. 1d and Supplementary information, Fig. S2. tSNE: t-distributed stochastic neighbor embedding. Note: the $40\ \mu\text{m}$ filtering step likely limits capture of the large Sertoli cells. **c** tSNE plot of single cell transcriptome data with cells colored based on their donors of origin, as indicated on the figure key. **d** Expression patterns of selected markers projected on the tSNE plot. Red indicates high expression and gray indicates low or no expression, as shown on the figure key. Top row represents somatic/niche cell markers; bottom three rows are representative germ cell markers. For each cell type, we show one marker in the main figures and a gallery in supplementary information, Fig. S2

distinct molecular signatures (4594 genes) suggesting striking transcriptional changes during meiotic entry and exit, but only gradual changes during meiosis. Notably, several RNA binding proteins were upregulated at zygotene/early pachytene, and some HOX genes (e.g., *HOXB4* and *HOXC6*) showed late pachytene-specific expression (Fig. 4c, d).

We observed a dynamic expression pattern of DMRT and SOX family members (Fig. 4e): Consistent with their role in meiotic entry inhibition in mice,^{26,27} *DMRT1* and *SOX4* were only expressed in pre-leptotene cells. Although the function of *DMRTC2* and *DMRT3* is largely unknown, *Sox30* knockout causes murine germ cell development arrest at round spermatid stage, and reduces expression of *Sox5*.^{28,29} Overall, our data are consistent with findings in mice, but also provide evidence for candidate genes with human-specific functions during meiosis.

Identification of known and novel spermatogonial stem cell states To further characterize the spermatogonial ‘States’, we re-clustered the early germ cells Clusters 1–2 (Fig. 5a). This analysis yielded five distinct clusters: while four showed high similarity to the clusters/states previously described (States 1–4 in ref.¹⁰) an additional state, hereafter termed ‘State 0’ was identified (Supplementary information, Table S4). None of the clusters/states (including State 0) consisted of cells derived from a particular donor or within a specific cell cycle phase (Supplementary information, Fig. S5a–d). Pseudotime analysis revealed a wave-like progression from State 0 to State 4, and clustering analyses defined gene expression signatures associated with each State (Fig. 5b; Supplementary information, Table S5). Notably, we observed a striking shift in transcriptional programs between State 1 and State 2, dominated by expression of cell cycle/proliferation genes (e.g., *MKI67*), suggesting that this transition represents a critical developmental node (see Discussion).

While both State 0 and State 1 cells co-express many key stem cell signaling factors and TFs (Fig. 5c, d), our analyses identify 490 genes that are either most highly expressed, or specifically expressed, in State 0 (e.g., *PIWIL4*, *EGR4*, *TSPAN33*, *PHGDH*, *PPP1R36*, *ICA1L* (Fig. 5e, f)). Known early SSC markers (*ID4*, *FGFR3*, *TCF3* and *UTF1*) were expressed in States 0 and 1, whereas known markers of differentiation (*KIT*) or proliferation (*MKI67*) were specifically expressed during or after State 2, suggesting State 0 and State 1 may represent two distinct quiescent SSC states. Interestingly, most State 0 cells displayed low expression of *ST3GAL2*, the enzyme that catalyzes the formation of SSEA4 (Supplementary information, Fig. S5g), suggesting that State 0 cells do not express this spermatogonial cell surface marker.

RNA velocity analysis and chromatin profiling suggest SSC plasticity

Next, we applied RNA ‘velocity’ analysis, a computational approach that utilizes nascent transcription in scRNA-seq datasets to infer developmental trajectories.¹¹ Here, the ratio of unspliced to spliced reads for each transcript is used as a proxy measurement of new transcription. By comparison with steady state (spliced) transcripts in the other cells, a velocity vector

representing the future transcriptional state of each individual cell can be defined. Within each cell of the tSNE plot (Fig. 6a), the amplitude and direction of the vector reflects a transcriptional trajectory. This analysis revealed two unexpected features. First, within the State 0 cluster, we observed two sub-populations: one proximal to State 1, bearing long vectors, indicating an apparent progression towards State 1—and a second sub-population lacking long vectors. This pattern suggests that the former cell sub-population is actively progressing/committing towards State 1, in response to specific developmental cues/signals, while the latter is not. Second, we also observed a sub-group of State 2 cells displaying long velocity vectors pointing back toward State 1. This forward and backward movement between States 0–1–2 raises the possibility that human spermatogonia display dynamic plasticity and metastable/uncommitted behaviors.

We then explored whether methylation or chromatin status may provide further evidence of plasticity. First, although $> 8,000$ genes show differential expression during spermatogonial development and spermiogenesis (Fig. 3b), we observed almost no differences in the DNAm profiles of SSEA4-enriched human SSCs and that of mature human sperm—which parallels a similar finding in mice.^{10,30} Thus, no DNAm barrier exists that might deter spermatogonial de-differentiation. Next, we profiled open chromatin from differentiating/committed spermatogonia (enriched using c-KIT), and compared it to profiles of self-renewing SSCs (enriched with SSEA4) (Fig. 6b). Notably, the open chromatin maps of c-KIT or SSEA4-enriched spermatogonia were highly similar ($r > 0.83$), and their nearest peak summits were typically overlapping (within a distance of ~ 120 bp), indicating that very few changes in the open chromatin landscape occurred during the commitment of undifferentiated SSEA4⁺ SSCs into differentiating c-KIT⁺ spermatogonia, in spite of the activation and repression of hundreds of genes. Taken together, the scRNA-seq analysis, the RNA velocity trends and evidence derived from the analysis of open chromatin landscape and DNAm are consistent with the proposal that SSCs follow a developmental progression involving 5 sequential transcriptional states characterized by a ‘flat’ chromatin/DNAm landscape, strongly suggestive of dynamic behaviors of spermatogonial cells (Fig. 6c).

The adult State 0 is most similar to infant germ cells

To assess whether State 0 may represent the earliest/naïve SSC in adults, we profiled testicular cells from infants (12–13 months old). After QC filtering, we obtained ~ 1300 single cells and assigned cell identities based on known markers. This analysis identified four somatic cell types, i.e., Sertoli, Leydig, endothelial and macrophage (Fig. 7a, b; Supplementary information, Fig. S7), and one tight cluster of 37 germ cells. Comparison to adult SSC States via tSNE and pseudotime analysis, positioned the infant germ cells adjacent to adult State 0, at the ‘beginning’ of the developmental trajectory (Fig. 7c). Moreover, most State 0 markers were highly expressed in infant germ cells (Fig. 7d). A small set of factors (e.g., *TBX3*, *HOXA3*) showed specific expression in infant spermatogonia,³¹ which may specify their germline identity. In addition, transcriptomic data of infant somatic cells should provide a useful resource for future analysis.

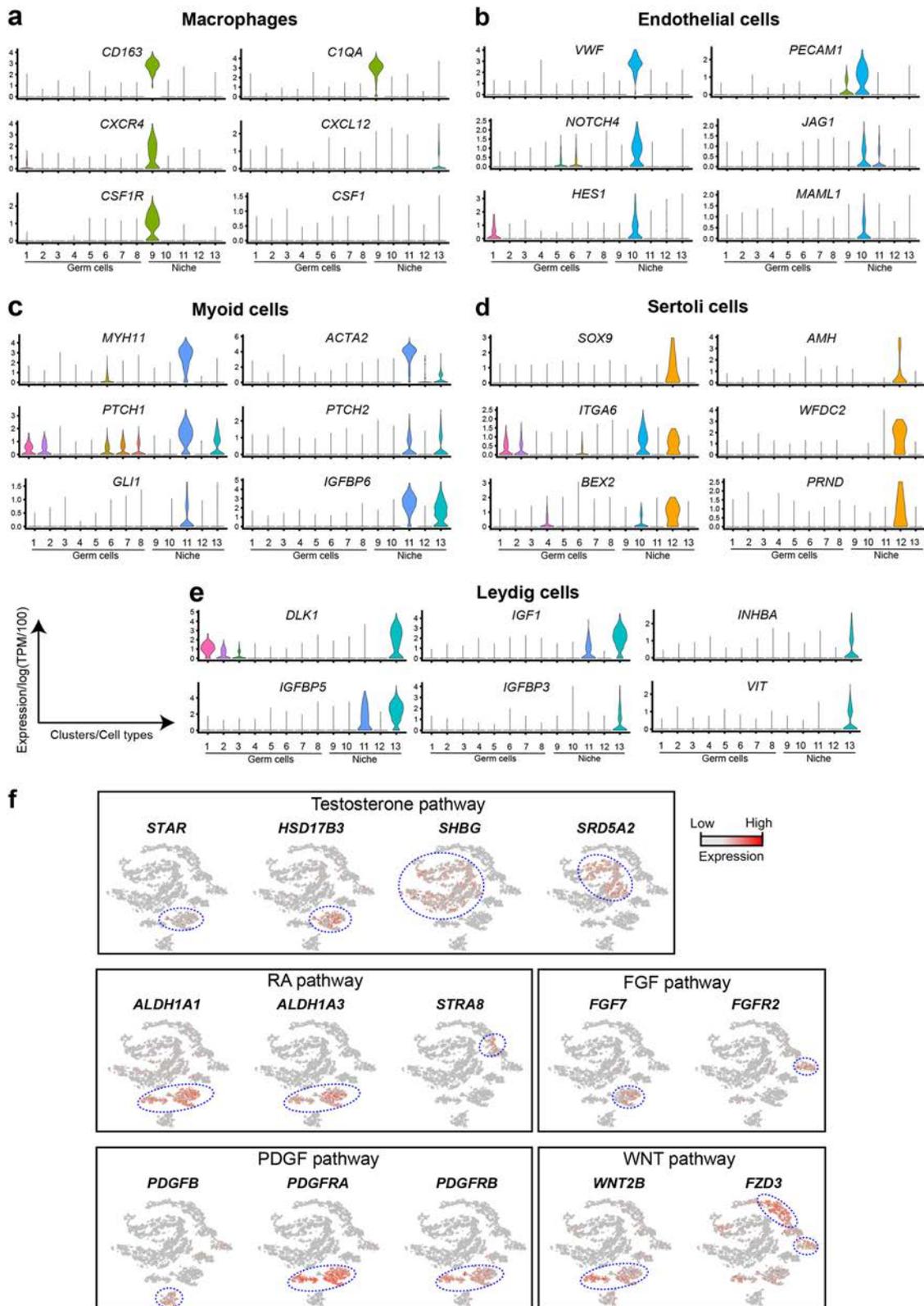


Fig. 2 Expression patterns of representative genes marking niche cells, and Niche-Germline interactions. **a** Expression patterns (violin plot) of macrophage-specific genes across the 13 different Clusters (Clusters 1–8 = germ cells; Clusters 9–13 = Niche/somatic cells). **b** Expression patterns (violin plot) of endothelial cell-specific genes across the different clusters. **c** Expression patterns (violin plot) of myoid cell-specific genes across the different clusters. **d** Expression patterns (violin plot) of Sertoli cell specific genes across different clusters. **e** Expression patterns (violin plot) of Leydig cell specific genes across different clusters. **f** Relative expression levels of representative genes from different key signaling pathways projected onto the tSNE plot from Fig. 1b. Stage-specific expression is highlighted by blue dotted circles

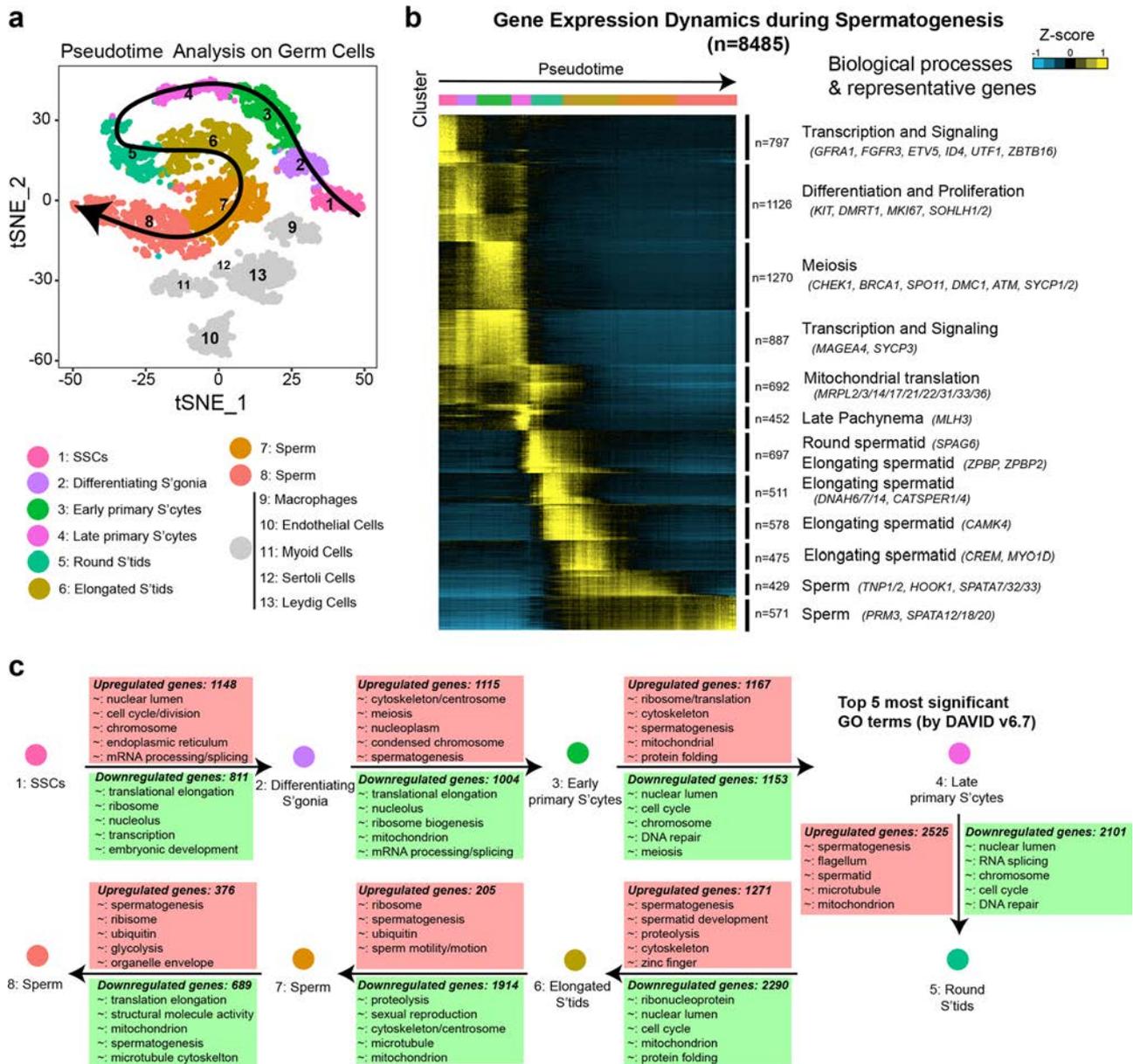


Fig. 3 Gene expression dynamics during spermatogenesis. **a** Pseudotime analysis on germ cells (Clusters 1–8). Cluster 1 represents the start of pseudotime, with Cluster 8 at the end. **b** K-means clustering of genes exhibiting differential expression ($n = 8485$) across germ cell populations. Note: each row represents a gene, and each column represents a single cell, with columns/cells placed in pseudotime order as defined in Fig. 3a and depicted by a thick colored line (top, color code as in Fig. 3a). Differential gene expression levels utilize a Z score, which represents the variance from the mean, as defined on the color key in the right top corner. **c** Differentially-expressed genes and associated GO terms (using DAVID v6.7) characteristic of germ cell developmental transitions, based on the 8 germ cell Clusters represented in Fig. 2a. The 5 most significant up-regulated GO terms are annotated in pink boxes, and down-regulated GO terms in green boxes. GO: gene ontology

Validation of SSC States via sequential mRNA fluorescence in situ hybridization

For validation, we performed sequential single molecule RNA fluorescence in situ hybridization (seqFISH)³² on 5 key genes (Fig. 8a; Supplementary information, Table S6). In cells expressing moderate/high *TCF3* (State 0 and State 1), only 27% (9/33) displayed high expression of *PIWIL4* (State 0 marker) and *ETV5/LITD1* (State 1), while the remainder expressed either *PIWIL4* (27%) or *ETV5/LITD1* (39%). This yielded significant non-overlap between State 0 and State 1 markers (hypergeometric test; $p = 0.03$), consistent with scRNA-seq data.

The State 0 marker TSPAN33 shows co-localization with high FGFR3

We next sought to enrich State 0 cells using cell surface markers, and assess their SSEA4 status. Here, we used TSPAN-family receptors, which show a strong enrichment in State 0 (e.g., TSPAN33; Fig. 5f). Flow cytometry analysis of cells expressing high levels of the SSC marker FGFR3 (peaks in State 0, present in State 1; Fig. 5d), show that FGFR3^{high} cells have high TSPAN33 but low SSEA4 (Fig. 8b)—characterizing State 0 as FGFR3^{high} TSPAN33^{high} SSEA4^{low}.

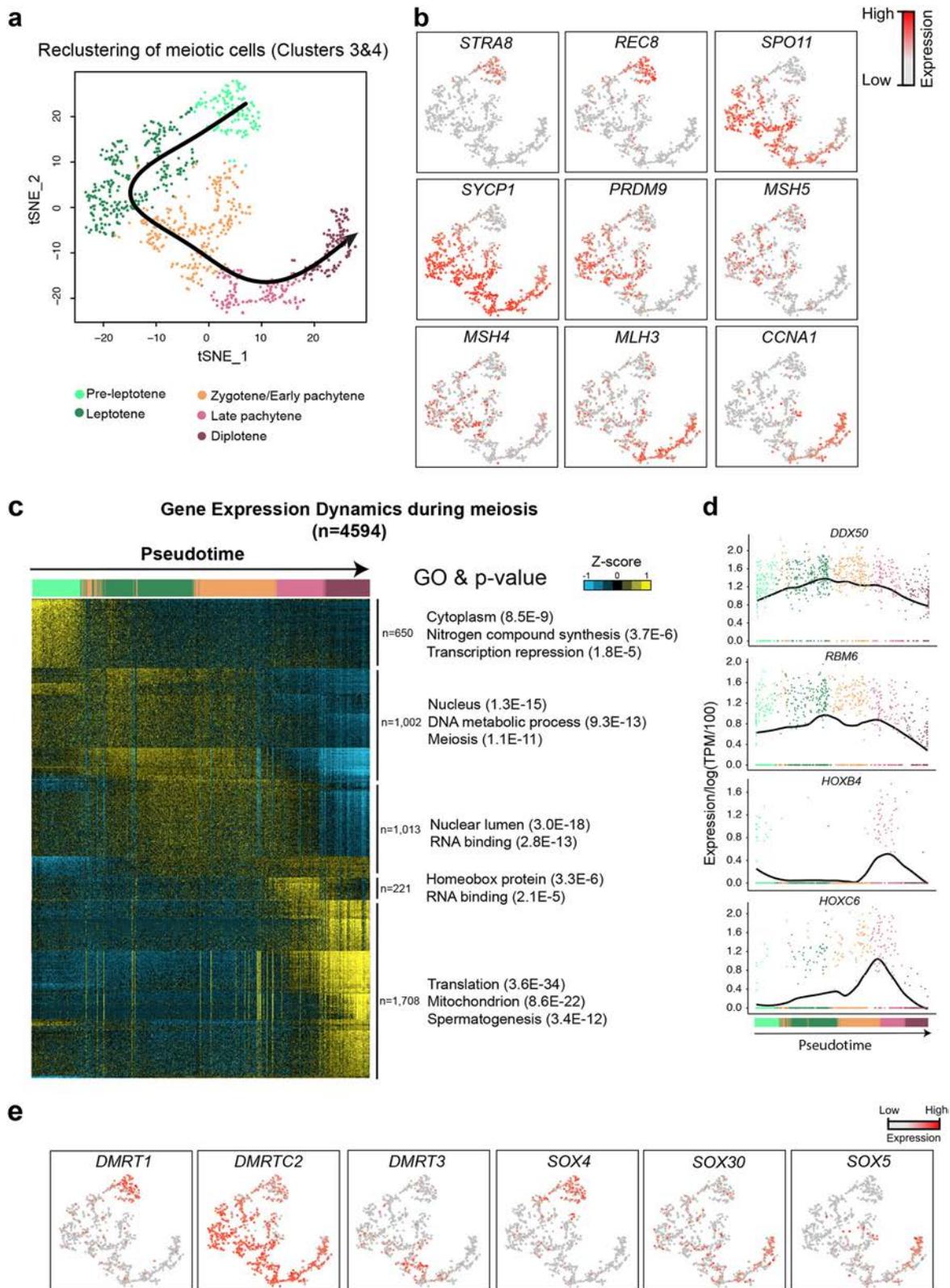
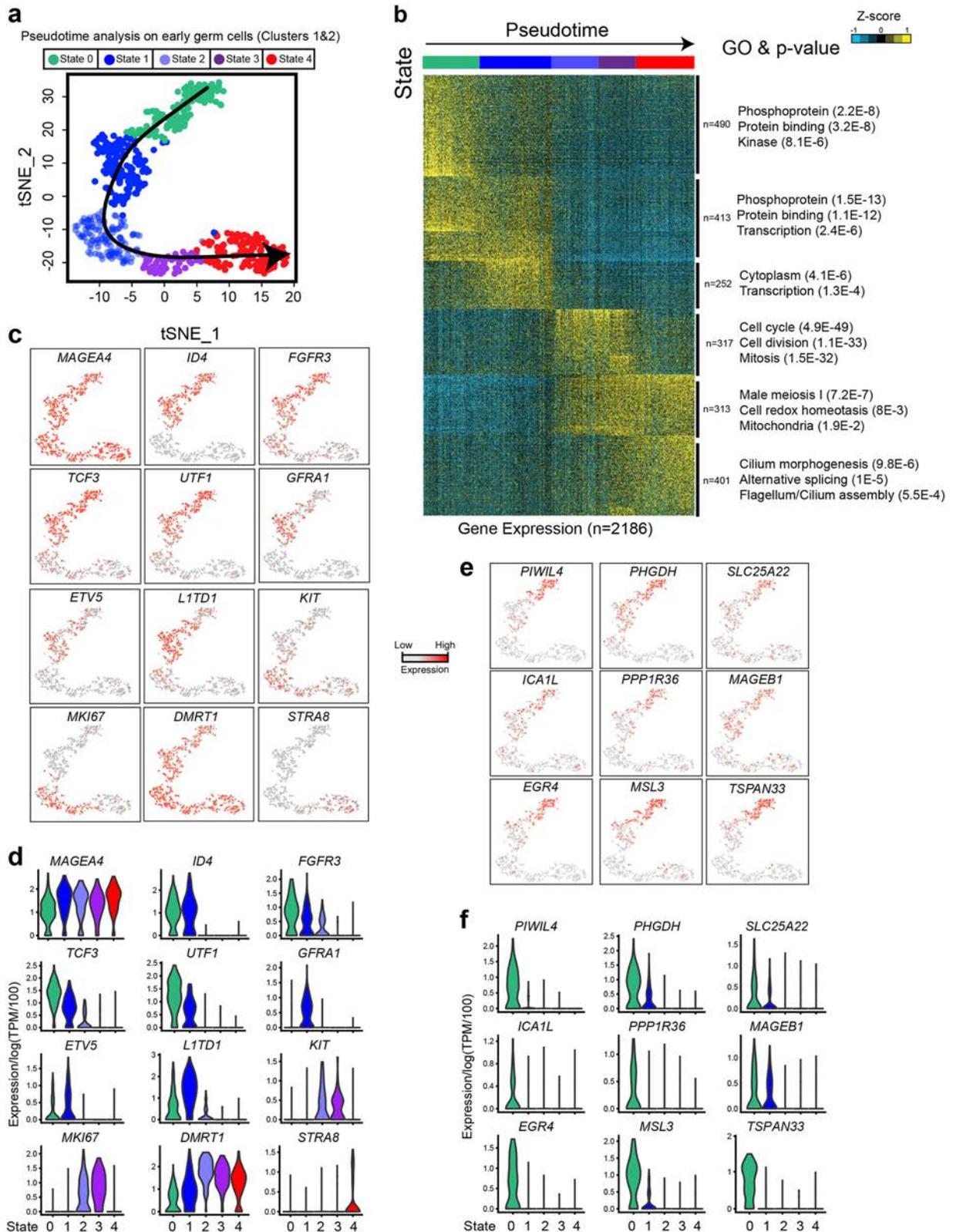


Fig. 4 Gene Expression Dynamics during Meiosis. **a** Focused analysis (tSNE, clustering and pseudotime ordering) of the cells from Clusters 3 and 4 (from Figs. 1b and 3a) reveals developmental progression during meiosis I. **b** Expression patterns of known meiotic markers projected onto the tSNE plot. Red indicates high expression and gray indicates low or no expression, key on figure. **c** K-means clustering of genes exhibiting differential expression ($n = 4594$) during meiosis I. Note: each row represents a gene, and each column represents a single cell, with columns/cells placed in pseudotime order as defined in **a** and depicted by a thick colored line (top, color code as in **a**). Gene expression levels utilize a Z score, which depicts variance from the mean, as defined on the color key in the right top corner. **d** Expression levels of representative genes during meiosis progression. x-axis represents pseudotime (as defined on **a**), and y-axis represents gene expression levels. **e** Expression patterns of key transcription factors during meiosis, with their expression projected onto the tSNE plot (**a**)



In situ observation of early SSC States via protein immunofluorescence
We characterized the protein expression of early spermatogonial markers (UTF1, GFRA1, FGFR3, and TCF3)³³ that exhibit differential expression across early States (0–1–2) by performing triple

immunofluorescence (IF) staining. *UTF1* expression peaked at State 0, and only partially overlapped with *GFRA1*, which peaks in State 1 (Fig. 5d). We observed that ~66% of cells located at the periphery of the seminiferous tubules expressed either *UTF1* and/or *GFRA1*, as previously reported.³⁴ Here, we distinguished two

Fig. 5 Identification of five discrete transcriptional states for SSCs. **a** Focused analysis (tSNE, clustering and pseudotime ordering) of Clusters 1 and 2 (from Fig. 1b and 3a) reveals five discrete cellular states (States 0 to 4) during SSC development. **b** K-means clustering ($k=6$) of genes exhibiting differential gene expression in States 0–4. Six gene clusters (termed S1–S6) were identified. Gene ontology associated with each gene block is shown on the right. Note: each row represents a gene, and each column represents a single cell, with columns/cells placed in pseudotime order (depicted by different colors on the top of the figure) as defined in **a**. Gene expression levels utilize a Z score, which depicts variance from the mean, as defined on the color key in the right top corner. **c** Relative expression levels of selected SSC markers projected on the tSNE plot represented in **a**. **d** Violin plots representing the expression levels of the selected markers shown in **c** in States 0–4 (x-axis). **e** Relative expression levels of selective State 0-specific markers projected on the tSNE plot represented in **a**. **f** Violin plots representing the expression levels of selective State 0-specific markers shown in **e** in States 0–4 (x-axis)

broad spermatogonial phenotypes, characterized by either $UTF1^{high}/GFRA1^{low}$ or $GFRA1^{high}/UTF1^{low}$, that recapitulate the temporal progression from State 0 to State 1. Furthermore, the spermatogonial markers *FGFR3* and *TCF3* were expressed in a subset of cells expressing either *UTF1* and/or *GFRA1*, but were never observed on their own (Fig. 8c). One simple explanation is that RNAs strongly expressed in State 0 (e.g., *UTF1*) produce a protein with a longer half-life than its RNA, and therefore the protein persists into the State 1 *GFRA1*-expressing cells—providing heterogeneity. As expected, the proliferating marker *MKI67* showed almost no overlap with *GFRA1* or *UTF1* (Fig. 8c and data not shown), suggesting that transition to the proliferative State 2 occurs with the loss of SSC markers, which is consistent with our computational analyses (Figs. 5 and 6c).

Finally, we further characterized the expression of genes specific to State 0 or State 1. Using the Human Protein Atlas resource (<http://www.proteinatlas.org/>) to review the pattern of 490 State 0 genes,³⁵ 16 candidate early SSC markers which displayed specific expression in cells located along the periphery of the seminiferous tubule were chosen. Triple IF staining with *UTF1* and *GFRA1* showed that each of these 16 markers is expressed in *GFRA1*-positive and/or *UTF1*-positive cells. Antibodies to *PHGDH* and *PPP1R36* display strong staining in $UTF1^{high}$ (State 0) cells, as predicted by the scRNA-seq analysis (Fig. 8d). Other State 0 markers showed expression in early spermatogonia (Fig. 8d; Supplementary information, Fig. S8b), an expected result for instances where the protein half-life is greater than that of the transcripts. In several instances, including *PIWIL4* and *MAGEB1*, we observed differences in staining intensity and/or sub-nuclear localization in cells co-expressing either $UTF1^{high}$ or $GFRA1^{high}$ marker (Fig. 8d; Supplementary information, Fig. S8b). These observations are consistent with the proposal that although State 0 and State 1 define transcriptionally discrete states, they likely represent metastable/heterogeneous cellular phenotypes that afford SSCs the ability to adapt to a dynamic niche environment and ensure homeostatic regulation within the testis.

DISCUSSION

Human adult spermatogenesis is a complex process, and a full understanding will involve the integration of multiple data types—including those from rodents, where genetic tools and SSC culturing systems are already available—to determine both shared and unique mechanism in mice and men. Here, we aimed to provide foundational scRNA-seq data of all cells contained within the normal human young adult testis, complemented by computational analysis and validation studies—to offer new insights into the regulation of male gametogenesis in humans.

Signaling features in the human testis niche

A major area of current interest involves communication between the niche and germline, and how these interactions mediate the changes observed during puberty and ageing. Here, we report new data that reveal potential differences between mice and men, and changes during development, for future functional investigation. First, for *CXCR4-CXCL12* signaling (for SSC homing to the niche), *CXCL12* was primarily expressed at the RNA level by Leydig

cells in humans rather than by Sertoli cells in mice. Furthermore, mouse SSCs express *Csf1r* for *Csf1* response, while human *CSF1R* expression appears to be specific to macrophages. Moreover, we identified novel markers for niche cells, like *WFDC2* and *PRND* for Sertoli cells, and *VIT* for Leydig cells. Beyond these examples, our datasets provide a resource for additional analyses of niche cells and niche-germline communication. Furthermore, our transcriptome data on infant testes concur that major differences exist between infant somatic cells and their counterparts in the adult (Supplementary information, Fig. S7), and exploration of the mechanisms mediating these changes prior to and during puberty will be an important future research focus.

Computational analysis of spermatogonial development and spermatogenesis

Here, we demonstrate that the dynamic and temporal trajectory of germline development could be reconstructed using scRNA-seq data and dedicated computational/analytical tools. The distribution of cell types for all three donors largely overlap during SSC stages and the early and mid stages of gametogenesis, establishing overall consistency between donors. However, modest differences were observed in late stages. Here, we note that the RNA content of maturing/mature sperm is both low and of poor integrity, a known and conserved property in mammals, conferring low number of genes/UMIs (unique molecular identifiers) in the maturing sperm datasets (Supplementary information, Fig. S1d). Therefore, although two distinct clusters of sperm were computationally obtained (Clusters 7 and 8), an alternative interpretation is that these sperm populations are similar, and simply contain different levels of RNA degradation/removal during this unique, transcriptionally inactive stage.

We also provide several new insights into spermatogonial development, most importantly the identification of a novel and early quiescent state, termed State 0. This interpretation is based on the properties/categories of the 490 differentially-regulated genes and the high similarities to the transcriptomic profile of infant germ cells. Moreover, State 0 cells (in contrast to State 1 cells) show low expression of the cell surface marker *SSEA4*, likely explaining why they escaped characterization in our prior study, which relied on *SSEA4*-mediated enrichment of testicular cells. Thus, we suggest that State 0 cells represent the undifferentiated and quiescent ‘reserve’ stem cell pool in the adult germline that is largely maintained from infants to adults.

Our data also call for a re-examination of prior work on human spermatogonial development. *GFRA1* marks early undifferentiated spermatogonia,³⁶ but exhibits a heterogeneous expression in human³⁴ and mouse³⁷ SSCs. Recently, using immunostaining, DiPersio et al.³⁴ proposed that ‘early’ (slow proliferating) $GFRA1^{high} UTF1^{-}$ SSCs progress to $GFRA1^{low} UTF1^{+}$, before committing to differentiation ($c-KIT^{+}$). However, our scRNA-seq data and pseudotime analysis suggest an alternative sequence of events—where *UTF1* precedes the expression of *GFRA1*—which, interestingly aligns with recent work in the mouse.³⁷ Beyond these two markers, we additionally provided data on hundreds of candidate spermatogonial markers (and validated 16 new protein markers) that define State 0 and State 1 SSCs in humans.

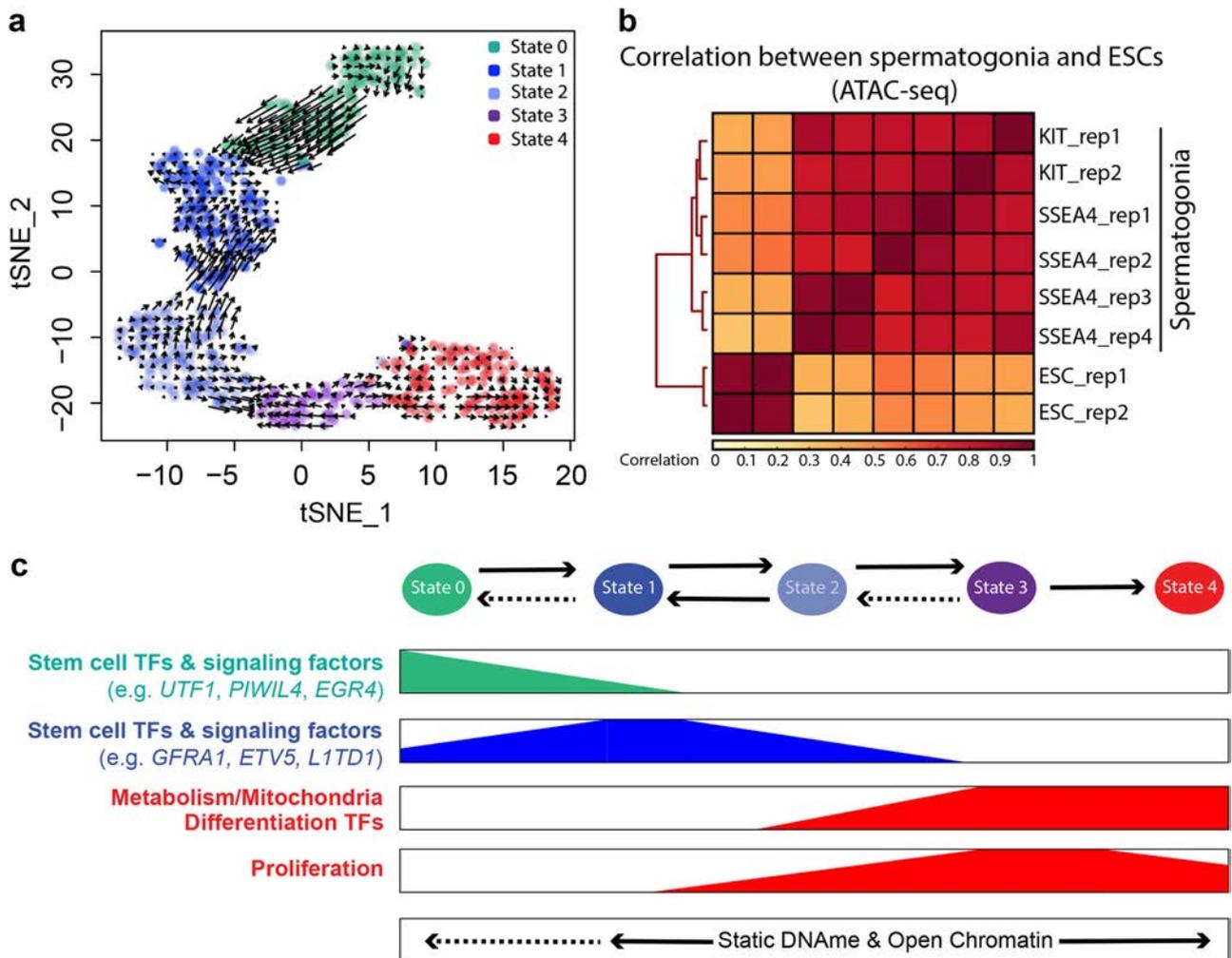


Fig. 6 Computational and molecular examination of spermatogonial plasticity. **a** Visualization of the RNA velocity analysis results on the tSNE plot of SSCs (see main text for details on vectors). **b** Heatmap and hierarchical clustering of ATAC-seq data from KIT⁺ spermatogonia (two replicates), SSEA4⁺ SSCs (four replicates) and ESCs (two replicates). Note: SSEA4⁺ SSC and ESC data are from ref.¹⁰ **c** Schematic summarizing the combinatorial gene expression programs and cellular events promoting five distinct SSC states (States 0–4) and depiction of the proposed spermatogonial dynamics/kinetics and behavioral plasticity of States with main cellular events and molecular pathways. Dotted arrows are speculative

Computational and molecular evidence for plasticity within early spermatogonia

Plasticity and stochastic behaviors within spermatogonial stem cell populations have been reported in several species including *Drosophila* and mice, in which differentiating spermatogonia can dedifferentiate and regain their early self-renewing properties.^{38–41} Our work provides two lines of evidence consistent with developmental plasticity in early human spermatogonia. First, RNA velocity analysis singled out a population of State 2 spermatogonia tending to ‘dedifferentiate’ into State 1-like cells. Notably, the transition from State 1 to State 2 is marked by an upregulation of proliferative markers—and thus might be a critical node for homeostasis by which developmental plasticity allows to balance early versus differentiating SSC populations.⁴² Second, we observe very limited changes in open chromatin and DNAm along the developmental trajectory of spermatogonia, which may enable transcriptional plasticity to take place by lowering epigenetic barriers to transcriptional changes, and dedifferentiation. Overall, we propose a spermatogonial developmental trajectory that involves 5 sequential transcriptional States, which generate moderately heterogeneous (metastable) proteomes, to enable State transitions and maintain a constant SSC pool; properties

which might be essential to maintain life-long fertility, and critical for the germline replenishment in case of damage.

A resource for future investigation of spermatogenesis
Recently, scRNA-seq has emerged as a highly useful approach for the study of human and mouse spermatogenesis.^{29,43,44} Here, our data reveals > 8000 genes that undergo significant differential regulation during male gametogenesis, and our results align well with a very recent scRNA-seq study of gametogenesis.⁴⁴ Beyond coding genes, our work has uniquely explored transposable elements (TE) and long non-coding RNAs—which are shown to display remarkable stage-specific expression. Of particular interest are the expression of active LTR12C/D/E, SVA_D and AluYa5 elements during early spermatogenesis, LTR10A and LTR40c elements during spermatogonial stages. We also described *XIST* expression during spermatogonial stages—which we show coincides with the selective attenuation of genes near the XIC (Supplementary information, Fig. S4), suggesting an unexpected role for *XIST* spreading and silencing in this process. Taken together, our datasets and analyses provide a comprehensive resource for the study of both niche cells and germline cells—including coding genes, TEs and lncRNA expression dynamics—

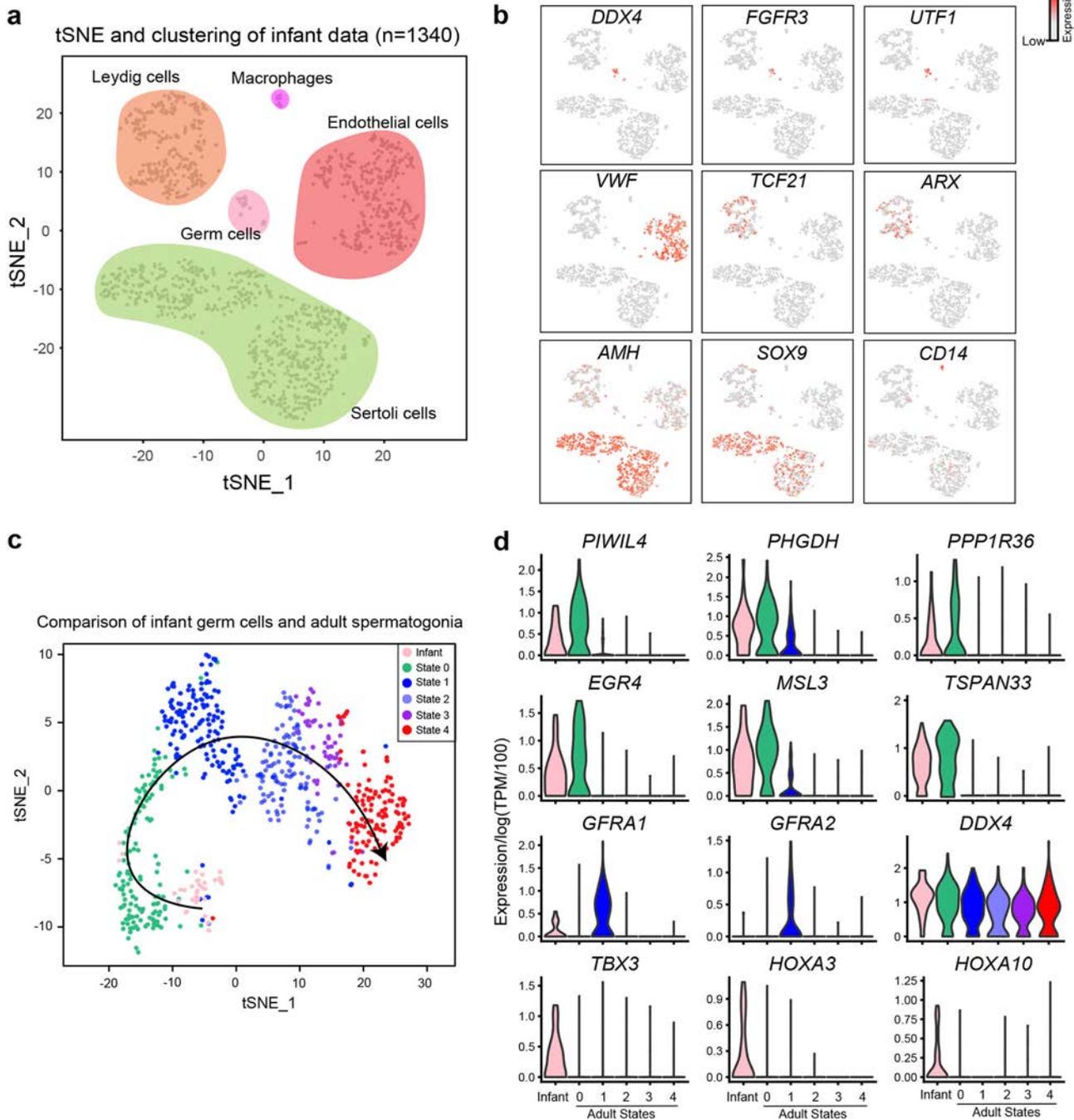


Fig. 7 Single cell RNA profiling from infant testis and comparison to adult scRNA-seq data. **a** tSNE and clustering analysis of single-cell transcriptome from infant testis ($n = 1340$). **b** Expression patterns of representative markers to help assign cell identities. **c** tSNE and pseudotime analysis of infant germ cells and adult spermatogonia. **d** Expression patterns (violin plot) of representative genes in infant germ cells and adult spermatogonia

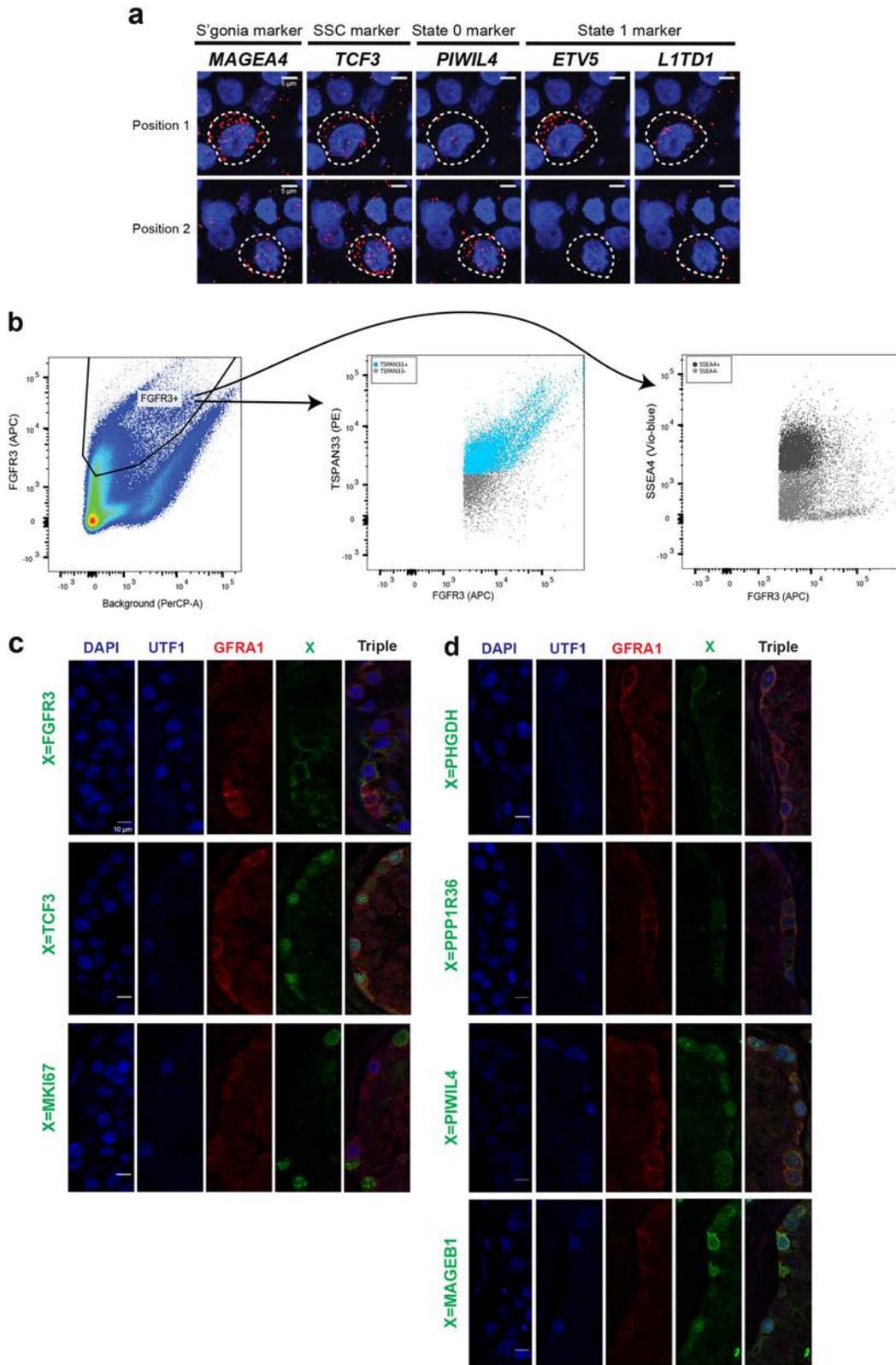
which will also serve as a useful reference dataset for comparisons to younger and older men, infertile men, and testicular cancer.

MATERIALS AND METHODS

Experimental model and subject details

Adult human testicular samples for scRNA-seq and immunostaining were from three healthy men (donor #1: 17 years old; donor #2: 24 years old; donor #3: 25 years old); sample for mRNA seqFISH was from a healthy man (donor #4: 23 years old). Infant testicular

samples for scRNA-seq were from two infant donors (13 months old). All six samples were obtained through the University of Utah Andrology laboratory and Intermountain Donor Service. Those samples were removed from deceased individuals who consented to organ donation for transplantation and research. Sample used for ATAC-seq was obtained through the University of Utah Andrology laboratory consented for research (IRB approved protocol #00075836: understanding the transcriptional and epigenetic dynamics in human spermatogonial stem cell self-renewal, proliferation and differentiation).



Sample storage by cryopreservation
Once collected, the pair of whole testis samples was transported to the research laboratory on ice in saline or Hank's Balanced Salt Solution (HBSS; GIBCO cat # 24020117) and processed within 1 h of removal by surgery. Around 90% of each testis was divided into

smaller portions (~0.5–1 g for each) using scissors and directly transferred into cryovials (Corning cat # 403659) in DMEM medium (Life Technologies cat # 11995073) containing 10% DMSO (Sigma-Aldrich cat # D8779), 15% fetal bovine serum/FBS (Gibco cat # 10082147) and cryopreserved in a Mr. Frosty Freezing container

Fig. 8 RNA and protein staining to validate state 0. **a** Sequential RNA FISH of SSC markers on tubular sections. Two SSCs are highlighted as representative examples. Blue is the DAPI signal; red detects RNA FISH signal for gene as indicated on the figure. White dashed line circles represent the cell boundaries. Scale bar: 5 μ m. **b** Expression patterns of FGFR3 (marks State 0 and 1), TSPAN33 (marks State 0) and SSEA4 (marks State 1) in testicular cells via flow cytometry. Human single testicular cells were used for staining with the markers indicated; non-stained cells were used as control for gating purposes. Left: FGFR3⁺ cells were identified and used for analysis in the middle and right panels. Middle: co-staining pattern between FGFR3 (x-axis) and TSPAN33 (y-axis), with blue as TSPAN33⁺ and red and gray as TSPAN33⁻. Right: co-staining pattern between FGFR3 (x-axis) and SSEA4 (y-axis), with black as SSEA4⁺ and gray as SSEA4⁻. **c** Immunolocalization of UTF1 (State 0 marker, in blue), GFRA1 (State 1 marker, in red) and FGFR3 or TCF3 or MKI67 (in green). Each combination of single or triple antigen (named in green on the left side) is represented by 5 panels. Scale bar: 10 μ m. **d** Immunolocalization of UTF1 (State 0 marker, in blue), GFRA1 (State 1 marker, in red) and 4 new candidate markers (in green). Each antigen (named in green on the left side) is represented by 5 panels. Scale bar: 10 μ m

(Thermo Fisher Scientific cat # 5100-0001) ensuring a slow controlled freezing rate at -80°C for overnight. Cryovials were then transferred to liquid nitrogen for long-term storage.

Sample fixation for immunostainings

Around 10% of the remaining testis tissues were incubated in 40 mL of 1 \times PBS containing 4% paraformaldehyde/PFA (Thermo Fisher Scientific cat # 28908) overnight at 4°C with agitation on a rotor (60 rpm). Fixed samples were then washed three times in cold PBS and stored in PBS at 4°C until processing for immunostaining.

Human adult testis sample preparation for single cell RNA sequencing

For each single cell sequencing experiment (technical replicate for one donor), ~ 5 cryovials were thawed in ~ 3 min. Tissues were washed twice in 1 \times PBS, and subjected to a standard two-step digestion procedure, as described previously.¹⁰ Briefly, testicular tissues were digested with collagenase type IV (Sigma Aldrich cat # C5138-500MG) for 5 min at 37°C with gentle agitation (250 rpm), then shaken vigorously and incubated for another 3 min. The tubules were sedimented by centrifugation at $200\times g$ for 5 min and washed with HBSS before digestion with 4.5 mL 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA; Invitrogen cat # 25300054) and 4 kU DNase I (Sigma Aldrich cat # D4527-500ku). The suspension was triturated vigorously three to five times and incubated at 37°C for 5 min. The process was repeated in 5 min increments for up to 15 min total. The digestion was stopped by adding 10% FBS (Gibco cat # 10082147). Single testicular cells were obtained by filtering through strainers with mesh size 70 μ m (Fisher Scientific cat # 08-771-2) and 40 μ m (Fisher Scientific cat # 08-771-1). The cells were pelleted by centrifugation at $600\times g$ for 15 min, and washed twice with 1 \times PBS. Cell number was measured using hemocytometer, and cells were then re-suspended in 1 \times PBS + 0.4% BSA (Thermo Fisher Scientific cat # AM2616) at the concentration of ~ 1000 cells/ μL , ready for single cell sequencing.

Human infant testis sample preparation for single cell RNA sequencing

We performed two technical replicates for the infant donor. A quarter of the testis was thawed in ~ 5 min. Tissues were washed twice in 1 \times PBS, and minced into small pieces for better digestion outcome. Tissues were then treated with trypsin EDTA for ~ 25 min at 37°C . The digestion was then stopped by adding 10% FBS (Gibco cat # 10082147). Single testicular cells were obtained by filtering through strainers with mesh size 70 μ m (Fisher Scientific cat # 08-771-2) and 40 μ m (Fisher Scientific cat # 08-771-1). The cells were pelleted by centrifugation at $600\times g$ for 15 min, and washed twice with 1 \times PBS. Cell number was measured using hemocytometer, and cells were then re-suspended in 1 \times PBS + 0.4% BSA (Thermo Fisher Scientific cat # AM2616) at the concentration of ~ 1000 cells/ μL , ready for single cell sequencing.

Single cell RNA-seq performance, library preparation and sequencing

scRNA-Seq was performed using the 10 \times Genomics system. Briefly, each experiment captured ~ 1500 single cells, in order to obtain $\sim 0.8\%$ multiplex rate. Cells were diluted following manufacturer recommendations, and mixed with 33.8 μL of total mixed buffer before being loaded into 10 \times Chromium Controller using Chromium Single Cell 3' v2 reagents. Each sequencing library was prepared following the manufacturer's instructions, with 13 cycles used for cDNA amplification. Then ~ 100 ng of cDNA were used for library amplification by 12 cycles. The resulting libraries were then sequenced on a 26 \times 100 cycle paired-end run on an Illumina HiSeq 2500 instrument.

Process of single cell RNA-seq data

Raw sequencing data were demultiplexed using the mkfastq application (Cell Ranger v1.2.1). Three types of fastq files were generated: I1 contains 8 bp sample index; R1 contains 26 bp (10 bp cell-BC + 16 bp UMI) index and R2 contains 100 bp cDNA sequence. Fastq files were then run with the cellranger count application (Cell Ranger v1.2.1) using default settings, to perform alignment (using STAR v2.5.4a), filtering and cellular barcode and UMI counting. The UMI count tables of each cellular barcode were used for further analysis.

Sequential RNA florescence in situ hybridization

Non-barcoded seqFISH (sequential FISH) probes were designed by targeting consensus of all constitutive exons (Supplementary information, Table S6) present in the masked hg38 human genome with 35-nucleotide (nt). All probes were blasted against the human transcriptome, and expected copy numbers of off-target probe hits were calculated using predicted RNA counts from RNA-seq dataset.¹⁰ Probes were then attached with one of the DNA hybridization chain reaction (HCR) initiator sequences (B1, B2, B3, B4 or B5) at 5' end with 4-nt 'ATAT' space in between. Initiator sequences were specific to genes in each round of hybridizations.

Non-barcoded seqFISH was performed by following the previous protocol.³² Human testis tissues were first perfused with RNase-free PBS, and embedded in 30% RNase free sucrose (VWR cat # 97061-430). After the tissues sank, they were frozen using a dry ice/isopropanol bath in OCT media and stored at -80°C . 15- μm sections were cut using cryotome and immediately placed on an aminosilane modified coverslip. The generated human testis sections mounted to coverslips (Thermo Scientific cat # 152450) were permeabilized at 4°C in 70% EtOH for 12-18 h. Tissue sections were further permeabilized by adding RNase-free 8% SDS (10% SDS Ambion cat # AM9822) for 20 min. Samples were rinsed with 70% EtOH to remove SDS, and air-dried. The hybridization chambers (Grace Bio-Labs cat # 621505) were adhered around the tissue sections. Then samples were washed once with 2 \times SSC (Invitrogen cat # 15557-036) diluted in Ultrapure water (Invitrogen cat # 10977-015), and hybridized with 2.5 nM probes per incubation for overnight at 37°C in Hybridization Buffer (50%

HB: 2× SSC, 50% Formamide (v/v) (Ambion cat # AM9344), 10% Dextran Sulfate (w/v) (Sigma cat # D8906), in Ultrapure water). Samples were washed in 50% Wash Buffer (50% WB: 2× SSC, 50% Formamide (v/v), 0.1% Triton-X 100 (Sigma X-100) in Ultrapure water) for 30 min at room temperature. While washing, aliquoted HCR hairpins (Molecular Instruments Inc.) were heated to 95 °C for 1.5 min and allowed to cool to room temperature for 30 min in the dark. The HCR hairpins were diluted to a concentration of 120 nM per hairpin in amplification buffer (2× SSC, 10% Dextran Sulfate (w/v)), and incubated with the samples for 45 min at room temperature in the dark. Following amplification, samples were washed in the 30% Wash Buffer (30% WB: 2× SSC, 30% Formamide (v/v), 0.1% Triton-X 100 in Ultrapure water) for 30 min to remove non-specifically bound hairpins. Samples were then stained with 5 µg/mL DAPI (Sigma cat # D8417) in 2× SSC and imaged as described below. After imaging, samples were digested with DNase I (10 units of DNase I, 1× buffer (Roche cat # 04716728001) in Ultrapure water) for 2 h at 37 °C on the microscope using custom made heat pad. Following DNase I treatment, the samples were washed with 30% WBT at 37 °C for 30 min, and hybridized with the following round of probe set for overnight with 2.5 nM probes per each in 50% HB at 37 °C on the microscope. Samples were then washed, amplified with HCR hairpins and imaged as before. The above steps were iterated at each hybridization round.

Following the last non-barcoded seqFISH, immunofluorescence was performed. The samples were washed with 1× PBS (Ambion cat # AM9624) for a few times, blocked with 5% BSA blocking solution (5% BSA (Gemini cat # 700–106 P), 1× PBS, and 0.3% Triton-X 100 in Ultrapure water), and then incubated at room temperature for 1 h. The primary antibody, Anti-beta Catenin (Abcam cat # ab6301), was 100-fold diluted in 1% BSA solution (1% BSA, 1× PBS, 0.3% Triton-X), incubated with the samples at room temperature for 3 h. The samples were then washed with 1× PBS for three times for 15 min each. The secondary antibody (anti-mouse Alexa Fluor 647 Invitrogen cat # A31571) was 500-fold diluted in 1% BSA buffer, and incubated with the samples at room temperature for 1 h. Samples were washed with 1× PBS for three times, stained with DAPI and imaged as described below.

Samples were imaged in an anti-bleaching buffer (14 mM Tris-HCl, pH = 8.0, 35 mM NaCl, 0.8% D-Glucose (Sigma cat # G7528), 100-fold diluted Catalase (Sigma cat # C3155), Pyranose oxidase with OD₄₀₅ of 0.05 (Sigma cat # P4234), and saturated amount of Trolox (Sigma cat # 238813) with the microscope (Leica, DMi8) equipped with a confocal scanner unit (Yokogawa CSU-W1), a sCMOS camera (Andor Zyla 4.2 Plus), 63× oil objective lens (Leica 1.40 NA), and a motorized stage (ASI MS2000). Lasers from CNI and filter sets from Semrock were used. Snapshots were acquired with 0.5 µm z steps across 15 µm with more than 10 positions per sample.

seqFISH signals were visualized using ImageJ software. Firstly, all images were aligned manually in xy and z by using DAPI channel signals. Each channel of HCR signals was background subtracted using ImageJ's subtract background function with rolling ball radius of 3 pixels. Images were applied by ImageJ's mean integral image filter with block radius of 3 pixels, and then contrasted. Each image was visualized with an overlay of DAPI signals of the first round of hybridization.

Immunostainings of testis tissues

The triple immunofluorescence stainings were performed on 5 µm formalin-fixed paraffin-embedded (FFPE) sections from portions of the testis from Donor 2 and 3 (24 and 25 years old respectively) following deparaffinisation, rehydration and heat-mediated antigen retrieval in 10 mM sodium citrate buffer solution (pH 6). After treatment with Superblock (PBS) Blocking Buffer (Thermo Fisher Scientific, cat # 37515) for 30 min, individual sections were

incubated overnight at 4 °C with a mix of three diluted antibodies (UTF1 (mouse monoclonal), GFRA1 (goat polyclonal) and a third rabbit polyclonal antibody (for antibodies details and dilutions, see the Table below). Antigen detection was conducted using the appropriate combination of Alexa Fluor 488, 555 and 647 secondary antibodies (all 1:500; Thermo Fisher Scientific, cat # A21202, cat # A21432, cat # A31573 respectively) for 2 h at room temperature in the dark. All primary/secondary antibodies were diluted in SignalBoost™ Immunoreaction Enhancer Kit (Calbiochem, cat # 407207–1KIT). After three washes in PBS, sections were incubated with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) (Roche, cat # 10 236 276 001) to facilitate nuclear visualization (dilution: 1 µg/mL). Specificity of the antibody staining was confirmed using the same protocol but with omission of primary antibodies. Following multiple washes in PBS, slides were mounted using Vectashield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA, cat # H-1000). Images were obtained under 25× objective (LD LCI PA 25× /0.8 DIC WD = 0.57 mm Imm Corr (UV)VIS-IR (Oil-Immersion) with a Zeiss LSM 780 Upright Multi-Photon Confocal Microscope and analyzed using Image J software (NIH, Bethesda, MD, USA).

Ab name	Antibody host	Ab ID	Dilution	Company
UTF1	Mouse monoclonal	MAB4337 (5G10.2)	(1:1000)	Millipore
GFRα1	Goat polyclonal	AF560	(1:25)	R&D systems
FGFR3	Rabbit mAb	C51F2 (#4574)	(1:50)	Cell signaling technology
Ki67	Rabbit polyclonal	ab16667	(1:200)	Abcam
MAGEB1	Rabbit polyclonal	HPA001193	(1:300)	Human protein atlas
PPP1R36	Rabbit polyclonal	HA077492	(1:2000)	Human protein atlas
CAMK2B	Rabbit polyclonal	HPA051783	(1:275)	Human protein atlas
PHGDH	Rabbit polyclonal	HPA24031	(1:500)	Human protein atlas
ERICH5	Rabbit polyclonal	HPA025070	(1:500)	Human protein atlas
PIWIL4	Rabbit polyclonal	HPA036588	(1:100)	Human protein atlas
TCF3	Rabbit polyclonal	HPA062476	(1:150)	Human protein atlas
APBB1	Rabbit polyclonal	HPA038521	(1:300)	Human protein atlas
C19orf81	Rabbit polyclonal	HPA060238	(1:100)	Human protein atlas
GPRC5C	Rabbit polyclonal	HPA029776	(1:135)	Human protein atlas
ICA1L	Rabbit polyclonal	HPA042507	(1:100)	Human protein atlas
LMNTD2	Rabbit polyclonal	HPA058474	(1:300)	Human protein atlas
MAGEC1	Rabbit polyclonal	HPA004622	(1:500)	Human protein atlas
TUBA1A	Rabbit polyclonal	HPA043684	(1:100)	Human protein atlas
MAP2K5	Rabbit polyclonal	HPA027347	(1:400)	Human protein atlas
HLA-DPA1	Rabbit polyclonal	HPA017967	(1:35)	Human protein atlas
SLC25A22	Rabbit polyclonal	HPA014662	(1:300)	Human protein atlas

Human c-KIT⁺ spermatogonia isolation using MACS

c-KIT⁺ cells were enriched using magnetic activated cell sorting (MACS) protocols (Miltenyi Biotec, Inc.). Single testicular cell suspensions were incubated with anti-c-KIT microbeads (Miltenyi Biotec cat # 130-098-571) at 4 °C. Following microbead binding, cells were re-suspended in autoMACS running buffer (Miltenyi Biotec cat # 130-091-221) and ran through LS columns (Miltenyi Biotec cat # 130-042-401) placed in a magnetic field. Columns were rinsed three times with buffer in autoMACS running buffer (Miltenyi Biotec cat # 130-091-221) before being removed from the magnetic field. MACS running/separation buffer (Miltenyi Biotec cat # 130-091-221) was then applied to the column before magnetically-labeled cells were flushed out by firmly pushing the plunger into the column. Cells were then centrifuged and re-suspended to a desired concentration.

ATAC-seq library preparation and sequencing

The ATAC-seq libraries were prepared as previously described⁴⁵ on ~30k sorted KIT⁺ spermatogonia, SSEA4⁺ SSCs or cultured ESCs.¹⁰ Briefly, collected cells were lysed in cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂ and 0.1% IGEPAL CA-630) and the nuclei were pelleted and resuspended in Transposase buffer. The Tn5 enzyme was made in-house and the transposition reaction was carried out for 30 min at 37 °C. Following purification, the Nextera libraries were amplified for 12 cycles using the NEBnext PCR master mix (NEB cat # M0541L) and purified using the Agencourt AMPure XP-PCR Purification (Beckman Coulter cat # A63881). All libraries were sequenced on a 125-cycle paired-end run on an Illumina HiSeq 2500 instrument.

Flow cytometry analysis

Cells were analyzed by flow cytometry using Aria Analyzer. For FGFR3 staining, cells were firstly incubated with anti-FGFR3 antibody (mouse monoclonal; Santa Cruz cat # sc-13121), washed and then incubated with Alexa Fluor-647 (anti-mouse; Thermo Fisher cat # Z25008); for TSPAN33 staining, cells were incubated with TSPAN33 PE-conjugated antibody (R&D Systems cat # FAB8405P-015); for SSEA4 staining, cells were incubated with SSEA4 VioBlue-conjugated antibody (Miltenyi Biotec cat # 130-098-366). Gating was based on unstained and single stained samples. FACS data were analyzed using FlowJo software (Ashland).

Quantifications and statistical analysis

Cell type identification and clustering analysis using Seurat program. The Seurat program (<http://satijalab.org/seurat/>, R package, v.2.0.0) was firstly applied for analysis of RNA-Sequencing data. To start with, UMI count tables from each replicates and donors were loaded into R using Read10X function, and Seurat objects were built from each experiment. Each experiment was filtered and normalized with default settings. Specifically, cells were retained only when they had greater than 500 genes expressed, and less than 20% reads mapped to mitochondrial genome. We first ran t-SNE and the clustering analysis for each replicate, which resulted in similar t-SNE map. Next, to minimize variation between technical replicates, we normalized and combined technical replicates from the same donor using the 10x Genomics built-in application from Cell Ranger “cellrange aggr”. Data matrices from different donors were then loaded into R using Seurat. Next, cells were normalized to the total UMI read count as well as mitochondrial read percentage, as instructed in the manufacturer’s manual (<http://satijalab.org/seurat/>). Seurat objects (matrices from different donors) were then combined using RunCCA function. t-SNE and clustering analyses were then performed on the combined dataset using the top 5000 highly variable genes and PCs 1–15, which showed most significant *p*-values. Given the low number of Sertoli cells (underrepresented

due to size filtering), the initial clustering analysis did not identify them as a separate cluster. We performed deeper clustering of somatic cells, identified the Sertoli cell cluster, and projected it back to the overall clusters, which resulted in 13 discrete cell clusters. Correlation of different replicates was calculated based on average expression (normalized UMIs by Seurat) in each experiment.

Pseudotime and clustering analysis. Germ cells (Clusters 1–8) from t-SNE plot were used for pseudotime analysis by slingshot (<https://github.com/kstreet13/slingshot>, R package, v0.1.2–3). Cluster 1 (SSCs) was used as start, cluster 4 (secondary spermatocytes) as middle, and cluster 8 (sperm) as end of pseudotime. After pseudotime time was determined, gene clustering analysis was performed to determine the fidelity of pseudotime. Here, cells (in columns) were ordered by their pseudotime, and genes (in rows) were clustered by k-means clustering using Cluster 3.0. Different k-mean numbers were used to reach the optimal clustering number. Genes within each gene clusters were then used to perform Gene Ontology analysis by David (v6.7).

Transposable element and lncRNA analysis. First, gtf files for TEs and lncRNAs were downloaded from UCSC and Incipedia, respectively. TE gtf was treated and filtered using the same approach as described.⁴⁶ These gtf files were then used to replace the default gtf files (for genes) in Cell Ranger, and UMI count tables were generated using the same approach as described above. For downstream analysis, TE and lncRNA expression patterns were cast to the gene expression based clustering and pseudotime.

Reclustering of spermatogonia/SSCs (Cluster 1 and 2). We parsed out cells in Cluster 1 and Cluster 2 (total number *n* = 614), and loaded their gene expression matrices into R through Seurat. Clustering and t-SNE analyses were performed, and a small cluster (containing 24 cells) was identified as outlier and excluded from further analysis. The remaining cells (*n* = 590) were re-clustered and analyzed using t-SNE using the top 5000 highly variable genes and PCs 1–5, which showed the most significant *p*-values. Pseudotime was performed as mentioned above using slingshot (v0.1.2–3).

Cell cycle analysis. Cell cycle analysis was performed using scran program (<https://bioconductor.org/packages/3.7/bioc/vignettes/scran/inst/doc/scran.html>, R Package; v1.6.5). Briefly, cell cycle genes were obtained from scran program, and their expression in States 0–4 were loaded into scran. Cell cycle phases (G1, S, and G2/M) were then assigned to each single cell.

Regulon analysis. Regulon analysis was performed using SCENIC program (<https://github.com/aertslab/SCENIC>, R Package; v0.1.7). State 0–4 cells were used to generate the regulon activity score of transcription factors as instructed by their manual. The regulon activity scores were then projected onto the t-SNE plot based on gene expression levels.

RNA velocity analysis. Cell velocity analysis was performed using Velocity.R program (<http://velocity.org>, v0.5), as instructed.¹¹ Briefly, at first, Velocity used raw data to count the spliced (mRNA) and unspliced (Intron) reads for each gene, and generated a.loom file. Those.loom files were then loaded into R (v3.4) using read.loom.matrices function to generate count tables for splicing and unsplicing reads. To generate RNA velocity map for spermatogonia, splicing and unspliced reads from States 0–4 were further used, and coordinates of the cells in the t-SNE plot were also provided. Lastly, the RNA velocity map was projected onto the t-SNE plot.

ATAC-seq comparison analysis. SAM alignments were generated from the Illumina Fastq files aligned to human hg19 genome using Novocraft's novoalign aligner (<http://www.novocraft.com>) with the following parameters: `-o SMA -r ALL 50`. Peak calling was performed using macs2 (<https://github.com/taoliu/MACS>, v2.1.2.20160309) using the following settings: `-g 2.7e9 -call-summit -f BAMPE -nomodel -B -SPMR -extsize 200`. Generated bedgraph file was then transformed to bw format using UCSC bedGraphToBigWig application (v4). Correlation was generated using deepTools (v3) by firstly using multiBigwigSummary bins application (with default settings) and then plotCorrelation application (with the following parameters: `--skipZeros --removeOutliers`). Distance of peak summit was calculated using bedtool (<http://bedtools.readthedocs.io/en/latest/content/tools/makewindows.html>, v2.25.0) closestBed application.

Data and software availability

The accession number for all sequencing data reported in this paper is GEO: GSE120508. Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Bradley R. Cairns (brad.cairns@hci.utah.edu).

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AUTHOR CONTRIBUTIONS

B.R.C. and J.G. conceived the project, designed the genomics and analysis experiments, and advised on other sections. J.G. conducted genomics experiments and computational analyses with help from E.J.G. Sample acquisition was led by J.M. H. and R.K. with input from D.T.C., B.R.C., and J.G. Samples were transferred and processed by J.G., Y.G., X.N. and E.J.G. Y.T., J.Y., and L.C. led the design and execution mRNA seqFISH. C.L. provided HPA antibodies. A.G. contributed to project design and led protein validation efforts. H.M. and G.J.M. performed protein immunostaining. J. G., A.G., and B.R.C. wrote the manuscript, with input from all authors.

ADDITIONAL INFORMATION

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