Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression

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SUMMARY

Mammalian spermatogenesis contributes a constant production of large numbers of spermatozoa, which is achieved by a cyclically regulated program known as the seminiferous epithelial cycle. Sertoli cells, functionally unique somatic cells, create a microenvironment to support the continuous differentiation of germ cells especially through the formation of a blood-testis barrier (BTB). The BTB is essential for maintaining homeostasis in seminiferous tubules and opens transiently at stages VII-VIII to ensure constant differentiation of spermatogenic cells. However, it is poorly understood how the dynamic organization of BTB is regulated. In our current study, we find that the overexpression of a dominant-negative form of RARα (dnRARα) in Sertoli cells disrupts the BTB at stages VII-XII and causes the large-scale apoptosis of differentiating germ cells. These abnormal events are found to be associated with cyclical gene expression changes in Sertoli cells, which can be represented by abnormal activation and repression of genes showing peaks of expression during stages I-VI and VII-XII, respectively. We find that one such gene, *Ocln*, encoding a tight junction component, partly contributes to the BTB disruption caused by dnRARα. Taken together, our data suggest that the cyclical activation of RA signaling in Sertoli cells during stages VII-XII contributes to a periodic organization of the BTB through changes in stage-dependent gene expression.

KEY WORDS: Retinoic acid, Spermatogenesis, Cyclicity of Sertoli cell, Mouse

INTRODUCTION

Mammalian spermatogenesis is a highly organized stem cell system that can continuously give rise to large numbers of spermatozoa and this constant production requires a proper microenvironment created by the somatic and germ cells themselves. Intriguingly, spermatogenesis progresses in a cyclically regulated manner, known as the seminiferous epithelial cycle (Russell et al., 1990; Hess and Renato de Franca, 2008; Hogarth and Griswold, 2010; Sugimoto et al., 2012). Spermatogonia, the immature male germ cells containing a stem cell population, proliferate several times and then differentiate into meiotic spermatocytes. After meiosis, these cells sequentially proceed to round spermatid and elongated spermatid stages, and are finally released into the lumen as spermatozoa. During spermatogenesis, germ cells at different developmental stages form groups and synchronously differentiate. In the mouse testis, 12 germ cell associations, known as seminiferous epithelial stages I-XII, have been identified, and are arranged in order along the longitudinal axis of the tubule where they show spatial continuity based on the differentiation step.

Sertoli cells are morphologically and functionally unique somatic cells that have specialized roles in support of spermatogenesis. It is known that these cells cyclically change their gene expression in accordance with the continuous alteration of the seminiferous epithelial stages, although the regulatory mechanism

is largely unclear (Timmons et al., 2002; Yoshida et al., 2006; Johnston et al., 2008; Hasegawa et al., 2012). Sertoli cells play a pivotal role in maintaining the integrity of the seminiferous epithelium by forming a blood-testis-barrier (BTB) (Cheng et al., 2010; Mital et al., 2011). The BTB is composed mainly of tight junctions (TJs) between adjacent Sertoli cells such that it restricts the entry of molecules in the interstitial space into the adluminal compartment and is thus required to maintain homeostasis in the adluminal compartment. However, to ensure that preleptotene spermatocytes located on the periphery enter the adluminal compartment and initiate meiosis, the TJs need to be disassembled at around stages VII-VIII. Concomitantly, new TJs must be assembled beneath the spermatocytes to maintain the BTB. This stage-dependent regulation of TJs has been reported to be achieved by the post-translational regulation of the proteins required to form the TJs (Su et al., 2010). However, it still remains elusive how the dynamic organization of BTB is linked with the cyclical gene expression change in Sertoli cells.

Retinoic acid (RA) is a small lipophilic molecule synthesized from vitamin A that controls multiple biological processes. RA signaling is mediated through three retinoic acid receptors (RARs) in the nucleus (Rosenfeld et al., 2006) and is involved in both ligand-dependent activation and suppression of target genes (Hua et al., 2009). It is known that RA signaling is involved in the cycle of spermatogenesis (Hogarth and Griswold, 2010). When vitamin A is depleted, spermatogenesis is arrested at the spermatogonial stage and the administration of RA into vitamin A-deficient (VAD) mice induces synchronized spermatogenesis (van Pelt and de Rooij, 1990). Moreover, Sertoli cell-specific RARα-conditional knockout (CKO) mice show disrupted stage-dependent gene expression and a mild degeneration of the seminiferous epithelium (Vernet et al., 2006a). Although these results indicate that RA is important for the cyclicity of spermatogenesis, the gene disruption was conducted

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before an intact seminiferous epithelial cycle was established. Thus, it remains unclear how RA signaling is involved in the stage-specific function of Sertoli cells during steady state spermatogenesis.

In our current study, we have examined the impact of overexpression of a dominant-negative form of the RARα receptor on the function of Sertoli cell in adult testes and found that the stage-dependent gene expression changes in Sertoli cells are primarily regulated by the periodic activation of RA signaling during stages VII-XII, and that this gene expression change is required for organization of the new TJs. We suggest from these results that the periodicity in Sertoli cells is important for the constant production of spermatozoa by maintaining the BTB.

MATERIALS AND METHODS
Animals
The *nanos3*^{−/−} mice have been previously described (Suzuki et al., 2008). The wild-type C57BL6/j mice were purchased from CLEA Japan. All animals were maintained in accordance with National Institute of Genetics (NIG) guidelines, and all animal procedures were carried out with approval from the Committee for Animal Care and Use at the NIG. Vitamin A-deficient (VAD) mice were generated as previously reported (van Pelt and de Rooij, 1990). At 11-16 weeks, when body weights had decreased, each mouse was injected with vehicle or 0.5 mg retinyl acetate (Sigma) intraperitoneally. Retinyl acetate was dissolved in 25 µl ethanol and mixed with 75 µl corn oil.

Preparation and injection of lentivirus
The packaging plasmids *pCAG-HIVgp* and *pCMV-VSV-G-RSV-Rev*, and the expression plasmids *CSII-EF-MCS-IRES2*-Venus and *CSII-EF-Venus*, were kindly provided by Dr H. Miyoshi (RIKEN BRC, Japan). The *pCMX-hRARE* (dnRARα) construct was a generous gift of Dr A. Kakizuka (Saitou et al., 1994). For the construction of *CS-II-EF-hRARE-IRES-Venus*, the open reading frame (ORF) of hRARE was excised from *pCMX-hRARE* and ligated into the multi-cloning site of *CS-II-EF-MCS-IRES-Venus*. *pSicoR*, a small hairpin RNA (shRNA) vector (Ventura et al., 2004), was purchased from Addgene. The sense oligonucleotides for the construction of shRNAs are listed in supplementary material Table S1. Those target sequences were designed using pSicoOligomaker 1.5 (http://web.mit.edu/jacks-lab/protocols/pSico.html). Lentiviruses were prepared and injected into 6- to 8-week-old C57BL6/j mice as previously described (Hasegawa et al., 2012).

Real-time RT-PCR
Total RNAs were purified using an RNeasy Kit (Qiagen), and cDNA was synthesized using oligo(dT) primer and SuperScript III (Invitrogen) in accordance with the manufacturer's instructions. Real-time RT-PCR was then performed using SYBR Premix Ex Taq II (Takara) and an MJ Mini Thermal Cycler (BioRad). Signals were normalized using *Gapdh* expression. The primer pairs used in these experiments are listed in supplementary material Table S1.

Microarray
For microarray analysis, seminiferous tubules at stages I-III, IV-VI, VII-VIII and IX-XII were isolated using dissection microscopy as previously described (Kotaja et al., 2004). Total RNAs were purified using an RNeasy Kit (Qiagen), and microarray analysis was conducted as previously reported (Suzuki et al., 2012). The data were then processed and normalized with GeneSpring GX 10.0 software (Agilent). Our microarray data are deposited in Gene Expression Omnibus (GEO) under accession number GSE34758.

Histological analysis
After removal of the tunica albuginae, mouse testes were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and embedded in paraffin. For histological analysis, sections were stained using Hematoxylin and the periodic acid Schiff (PAS) method. Seminiferous epithelial stages were determined as described previously (Russell et al., 1990). For

immunohistochemistry, paraffin sections were immersed in unmasking solution (Vector Laboratories) or in Target Retrieval Solution (DAKO). This was followed by treatment with PBST (PBS with 0.1% Tween 20) containing 3% skimmed milk and an overnight incubation at 4°C with the following antibodies: chick ant-GFP (Aves), goat anti-Gata4 (Santa Cruz), rabbit anti-vimentin (MBL), rabbit anti-WT1 (Abcam), rabbit anti-ZO1 (Invitrogen), rat anti-occludin (Sanko Junyaku) and rat anti-TRA98 (Sada et al., 2009). The resulting signals were detected by incubation with an Alexa488- or Alexa594-conjugated (Molecular Probes) or horseradish peroxidase (HRP)-conjugated secondary antibodies (Aves). Sections were counterstained with DAPI and images were processed with Adobe Photoshop v10.

TUNEL assay
Following lentivirus injection, mouse testes were fixed and paraffin sections were prepared as described above. Sections of these tissues were then treated with 15 µg/ml Proteinase K for 10 minutes at 37°C and stained using the In Situ Cell Death Detection Kit (Roche) following the manufacturer's protocol. The sections were then stained with an anti-GFP antibody and counterstained with DAPI.

In situ hybridization
In situ hybridization was performed as previously described (Hasegawa et al., 2012). *Ptpre* (2.0 kb), *Serpina3a* (1.7 kb), *Dhcr24* (2.2 kb), *Dpysl4* (2.0 kb), *Rgma* (1.6 v), *Drd4* (1.1 kb), *Ltb4dh* (1.9 kb), *Tspan17* (1.3 kb), *Lgals1* (0.5 kb), *Stra6* (2.3 kb), *Stra8* (1.4 kb), *Gas6* (1.7 kb) and *Plat* (2.4 kb) were subcloned from testis cDNA by RT-PCR. The primer pairs used for each subcloning are listed in supplementary material Table S1. Digoxigenin (DIG)-labeled cRNA probes were synthesized with RNA Labeling Mix (Roche). Paraffin sections were hybridized with each DIG-labeled probe, and incubated with HRP-conjugated anti-DIG Fab fragments (Roche). The signals were detected using a Cy3-Tyramide Signal Detection Reagent (Perkin Elmer).

Biotin tracer experiment
Biotin solution was microinjected into testes as previously described (Takashima et al., 2011). The testes without tunica albuginae were fixed with 4% PFA overnight and embedded in paraffin. Distribution of biotin was detected with streptavidin-conjugated Alexa-488 (Molecular Probes).

Isolation of Sertoli cells
For the direct isolation of adult Sertoli cells from wild-type and *Nanos3*^{−/−} mice, testes were sequentially treated with 1 mg/ml collagenase in DMEM containing 25 U/ml DNaseI twice at room temperature for 10 minutes, and then with 2 mg/ml hyaluronidase and 1 mg/ml collagenase in DMEM containing 25 U/ml DNaseI at 32°C for 20 minutes with agitation. After filtration using a 35-µm filter, cells were suspended in PBS containing 2% fetal bovine serum (FBS) and 100 µg/ml propidium iodide, and isolated with a JASN Cell Sorter (Bay Bioscience). To confirm the purity of these preparations, isolated cells were seeded on a glass slide and immunostained with anit-WT1, anti-vimentin and anti-TRA98 antibodies.

Primary Sertoli cells were isolated and cultured as previously described (Hasegawa et al., 2012). The culture medium was changed at days 2 and 4, and Sertoli cells were stimulated with 1 µM retinoic acid (Sigma) at day 5 for 24 hours.

Statistics
All statistical analyses in this study were performed using Student's *t*-test. Statistical significance was defined as $P<0.05$.

RESULTS
Overexpression of dnRARα in Sertoli cells induced stage-specific defects in spermatogenesis
To investigate the importance of RA signaling in Sertoli cells, we utilized a dominant-negative form of RARα (dnRARα) that can bind DNA but not RA (Saitou et al., 1994). This variant suppresses RA signaling by competing with RARα for DNA-binding sites, although a canonical RA signaling-independent effect of
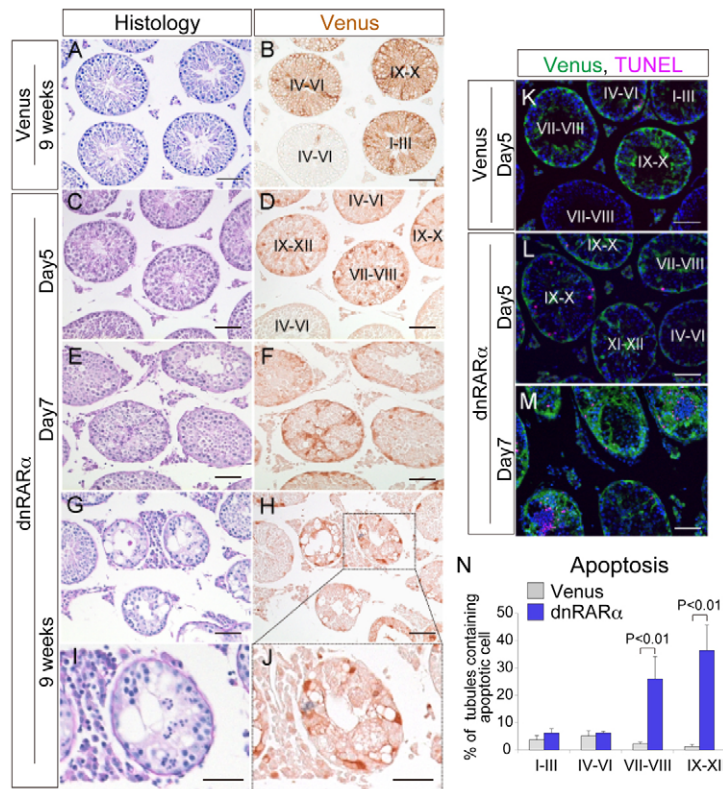
Fig. 1. Histological analyses of the abnormal seminiferous epithelium induced by the overexpression of dnRARα. (**A-J**) Sections derived from mouse testes injected with LV-Venus or LV-dnRARα were stained with PAS and Hematoxylin at 5 days, 7 days and 9 weeks post-injection. Serial sections were stained with a GFP antibody to detect transgene expression. I and J show higher magnification images of those in G and H, respectively. (**K-M**) TUNEL staining (magenta) and Venus immunostaining (green) of sections derived from testes injected with LV-Venus or LV-dnRARα. (**N**) Quantification of stage-dependent apoptosis in Venus-positive tubules (*n*=4). Error bars represent s.d. Scale bars: 80 μm in A-H,K-M; 40 μm in I,J.

dnRARα has also been reported (Andersen and Rosenfeld, 1995; Chen and Lohnes, 2005). We overexpressed dnRARα in Sertoli cells by injecting lentivirus containing this variant (LV-dnRARα) into seminiferous tubules (supplementary material Fig. S1; Fig. 1). We used a lentivirus containing Venus (LV-Venus) as a control. We confirmed specific infection of Sertoli cells, but not of germ cells upon lentivirus injection into seminiferous tubules by Venus reporter expression (supplementary material Fig. S1B,C), as also reported previously (Ikawa et al., 2002; Hasegawa et al., 2012). In the following experiments, we analyzed adult testes in which 50-90% of tubules include more than one transgene-positive Sertoli cell.

We examined first the extent to which spermatogenesis is affected by the overexpression of dnRARα in Sertoli cells. Transgene expression in Sertoli cells commenced on day 3 and reached a maximum level by day 5 post-injection (Fig. 1C,D). On day 7, degeneration of the seminiferous epithelium was evident and the seminiferous epithelial stages became indistinguishable (Fig. 1E,F). At 9 weeks post-injection, a massive level of germ cell loss was observed (Fig. 1G-J). To examine the underlying causes of this substantial germ cell loss, we assayed these cell populations for apoptosis by TUNEL staining. Interestingly, elevated apoptosis among spermatocytes and spermatids at day 5 post-injection was evident at stages VII-XII only (Fig. 1K,L,N). At day 7, the apoptosis of spermatogenic cells had spread to most of the Venus-positive tubules (Fig. 1M). These results suggest that RA signaling is important for the survival of differentiating germ cells by regulating the function of Sertoli cells, and that the germ cells at stages VII-XII are more susceptible to the overexpression of dnRARα in Sertoli cells.

During normal spermatogenesis, elongated spermatids are positioned in crypts within the Sertoli cells at stage VI and migrate to the luminal surface of the seminiferous epithelium at stage VII.

They are then released into lumen by the end of stage VIII. At day 5 post-injection of LV-dnRARα, however, 55.4% of Venus-positive tubules at stages VII-VIII still contained elongated spermatid localized near the periphery (supplementary material Fig. S2A,C,E) and 45.8% of Venus-positive tubules at stage IX abnormally contained elongated spermatids (supplementary material Fig. S2B,D,F), indicating that RA signaling in Sertoli cells is required for the proper localization and release of elongated spermatids. Collectively, these results suggest that activation of RA signaling in Sertoli cells is important for the survival and differentiation of spermatocytes and spermatids during stages VII-XII.

Overexpression of dnRARα in Sertoli cells disrupts the blood-testis barrier

Degeneration of the seminiferous epithelium and germ cell loss are often observed in mutant mice showing BTB abnormalities (Gow et al., 1999; Saitou et al., 2000; Xu et al., 2009). We thus hypothesized that overexpression of dnRARα in Sertoli cells might affect the BTB organization. Consistent with previously reported observations of mice showing BTB abnormalities, we found that Sertoli cells were abnormally detached from the periphery of stage VII-XII tubules at 5 days after LV-dnRARα injection (Fig. 2A-D). Next, we examined the permeability of the BTB using a biotin tracer, and found that biotin signals were detected in the adluminal space of LV-dnRARα-injected tubules during stages VII-XII, but not at the other stages or in LV-Venus injected tubules (Fig. 2E-H,M). Furthermore, the expression of zona occludens-1 (ZO1; Tjp1 – Mouse Genome Informatics), a peripheral adaptor protein of the TJs and a BTB marker (Wong et al., 2004), was reduced in stage VII-XII tubules, but was unchanged in stage I-VI tubules (Fig. 2I-L). These results suggest that the activation of RA signaling in Sertoli cells is required for the organization of the BTB during stages VII-XII.
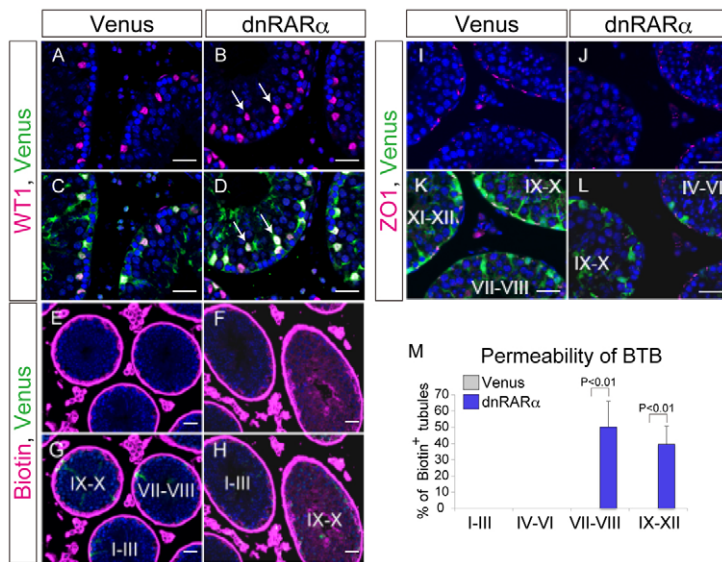
Fig. 2. Abnormal BTB regulation upon overexpression of dnRARα. (**A-D**) Immunostaining for WT1 (magenta) and Venus (green) using sections of mouse testes injected with LV-Venus or LV-dnRARα. Arrows indicate the nuclei of Sertoli cells that had detached from the periphery. Stages were determined by serial section staining with PAS and Hematoxylin. (**E-H**) Functional assessment of BTB. Distribution of biotin tracer (magenta) was visualized with streptavidin conjugated with Alexa488 and Venus (green) was detected with a GFP antibody. (**I-L**) Immunostaining for ZO1 (magenta) and Venus (green) on sections of testes injected with LV-Venus or LV-dnRARα. (**M**) Quantification of tubules showing biotin permeability in Venus-positive tubules (n=4). Error bars represent s.d. Scale bars: 40 μm.

Comprehensive identification of stage-dependent genes in Sertoli cells

Our in vivo study using dnRARα indicated that the changes in RA signaling in Sertoli cells might contribute to BTB regulation. To study more directly the mechanism of the stage-dependent disruption of the BTB by dnRARα, we investigated gene expression changes induced in Sertoli cells by dnRARα. For this purpose, a fuller understanding of stage-dependent gene expression changes in Sertoli cells during steady state spermatogenesis is required. Therefore, we performed comprehensive gene expression profiling of these cells in normal adult testes by microarray (Fig. 3). To identify genes that are highly expressed in Sertoli cells, we used cultured primary Sertoli cells, Sertoli cells directly isolated from both wild-type and germ-less *Nanos3⁻/⁻* testes in comparison with whole testes (Fig. 3A). For this experiment, we developed a method of purifying Sertoli cells using fluorescence-activated cell sorting (FACS) because of the low proportions of Sertoli cells in adult testes (supplementary material Fig. S3).

Most of the *Nanos3⁻/⁻* testicular cells showed a relatively high level of intrinsic fluorescence and high granularity (supplementary material Fig. S3A,C). By contrast, most of the testicular cells derived from adult wild-type testes showed low intrinsic fluorescence (R4) and low granularity (R3) (supplementary material Fig. S3B,D). Accordingly, we isolated cells showing high intrinsic fluorescence (R1) and high granularity (R2) as the fraction enriched in Sertoli cells (supplementary material Fig. S3A-C,E). The high purity of these Sertoli cell preparations after sorting was confirmed by positive immunostaining for both WT1 and vimentin, established markers of Sertoli cells, and negative staining for TRA98, a marker for germ cells from the spermatogonia to round spermatid stages (supplementary material Fig. S3F-H). We also purified RNA from cultured primary Sertoli cells, which showed >95% purity, and conducted microarray analysis with the three types of purified Sertoli cells. By comparison with the expression pattern in the whole testis, 5996, 5364 and 6987 genes showed a threefold higher expression in cultured Sertoli cells and in Sertoli cells directly isolated from wild-type and *Nanos3⁻/⁻* testes, respectively (Fig. 3A). Among these, we defined 8226 unique genes as highly expressed in Sertoli cells. Many of them were common among these samples and ~80% of those genes were included in more than two samples.

Next, to define novel stage-dependent genes in the seminiferous tubules, we isolated tubules at stages I-III, IV-VI, VII-VIII and IX-XII (Kotaja et al., 2004) and performed gene expression profiling by microarray. We thereby identified 377, 760, 956 and 806 genes showing expression peaks at stages I-III, IV-VI, VII-VIII and IX-XII, respectively, using a criterion of a greater than threefold change between any two stages (Fig. 3B). By comparing these genes with the 8226 genes found to be highly expressed in Sertoli cells, we finally identified 68, 116, 130 and 105 genes that are enriched in Sertoli cells and show expression peaks during stages I-III, IV-VI, VII-VIII and IX-XII, respectively (Fig. 3C,D). This list contained already reported stage-dependent genes, such as *Gas6*, *Gata1*, *Plat* and *Lgals1*, and we randomly selected eight genes for which we performed in situ hybridization analysis to confirm their stage-dependent gene expression in Sertoli cells (supplementary material Fig. S4). These results validated our microarray analysis. We now refer to this entire panel of 419 genes as 'stage-dependent genes in Sertoli cells', which corresponds to 5.1% (419/8226) of the highly expressed genes in these cells (Fig. 3C,D; supplementary material Table S2).

Regulation of stage-dependent gene expression in Sertoli cells by RA signaling

To investigate which stage-dependent genes are affected by the overexpression of dnRARα, we conducted microarray analysis. We isolated seminiferous tubules at stages I-III, IV-VI, VII-VIII and IX-XII from LV-dnRARα injected tubules at day 5 post-injection because the expression of the transgene is stronger after this period and the seminiferous epithelial cycle remains intact (Fig. 1C,D). We then compared the expression levels of the identified stage-dependent genes (Fig. 3) in the LV-dnRARα-injected tubules with those of control tubules by microarray (Fig. 4). Strikingly, ~40% of the genes showing peak expression during stages I-VI and VII-XII were upregulated or downregulated, upon overexpression of dnRARα. The gene expression change was most evident at stages VII-XII, suggesting that RA signaling is more strongly activated at stages VII-XII than at stages I-VI. The results of our microarray analysis were confirmed by qRT-PCR (supplementary material Fig. S5). We also examined expression changes of the stage-dependent genes in primary Sertoli cells after RA treatment by microarray.
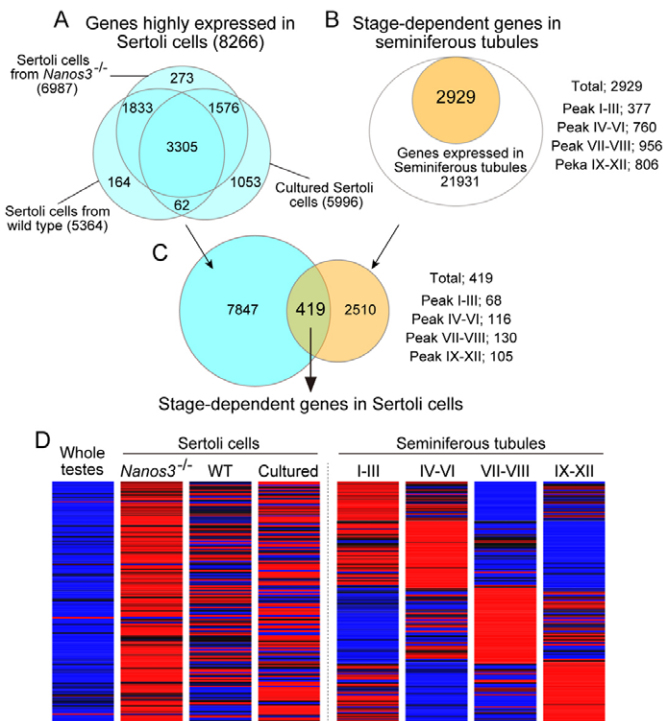
Fig. 3. Identification of stage-dependent genes in mouse Sertoli cells. (**A**) Identification of genes highly expressed in Sertoli cells. The transcriptome of three types of isolated Sertoli cells were compared with that of whole testes and 8266 genes that showed a threefold higher expression were thereby identified. (**B**) Identification of stage-dependent genes in the seminiferous tubules. The transcriptome of tubules at stages I-III, IV-VI, VII-VIII and IX-XII were compared. This approach revealed that 24,860 genes are expressed in the tubules, of which 2929 show a greater than threefold change between any two stages. (**C**) The genes found to be highly expressed in Sertoli cells and the stage-dependent genes in seminiferous tubules were compared, from which 419 common genes were defined as stage-dependent genes in Sertoli cells. (**D**) Heat map data demonstrating the expression patterns of the 419 stage-dependent Sertoli cell genes in the indicated samples. Red and blue represent higher and lower gene expression, respectively.

Fig. 4. Expression changes in the stage-dependent genes induced by overexpression of dnRARα. Seminiferous tubules at stages I-III, IV-VI, VII-VIII and IX-XII were isolated at 5 days post-injection with LV-Venus or LV-dnRARα. The expression levels of the stage-dependent genes were then compared by microarray. The proportion of stage-dependent genes showing each expression peak (indicated at the top) were up- or downregulated in stage-specific tubules (indicated at the bottom) following the overexpression of dnRARα. The total number of stage-dependent genes is indicated in brackets. (*n*=2).

Consistent with in vivo results, ~25% of the stage-dependent genes showing peak during stages I-VI and VII-XII were suppressed or activated (supplementary material Fig. S6A,B). When we compared the gene lists, we found that 51.8% (87/168) of genes affected by overexpression of dnRARα in seminiferous tubules overlapped with those that responded to the RA treatment in primary Sertoli cells, and 80.6% (87/108) of genes that responded to the RA treatment in primary Sertoli cells overlapped with those of genes that responded to dnRARα in seminiferous tubules (supplementary material Fig. S6C), suggesting that a considerable proportion of the genes affected by dnRARα was affected by suppression of RA signaling. Furthermore, the administration of vitamin A to VAD mice also resulted in similar expression changes of eight stage-dependent genes (supplementary material Fig. S7). Taken together, these results suggest that RA signaling is periodically activated during stages VII-XII and thereby induces stage-dependent gene expression in Sertoli cells by altering the expression of RA-responsive genes (supplementary material Fig. S8).
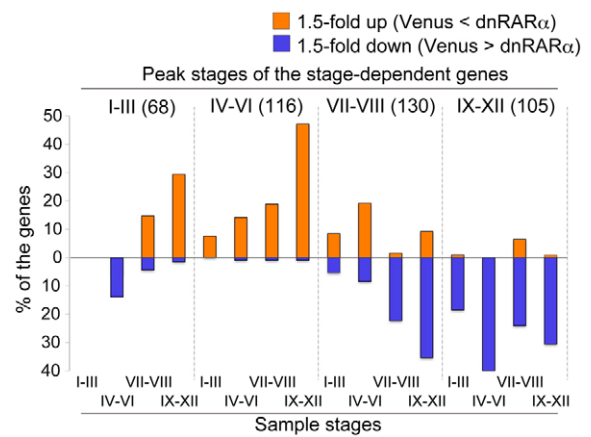
In vivo knockdown of occludin induces disruption of blood-testis barrier and apoptosis of germ cells

To identify the gene(s) responsible for disrupting the BTB organization, we screened our microarray data for tight junction-associated genes showing reduced expression in testes injected with LV-dnRARα. We thereby identified *Ocln*, which encodes the tight junction integral protein occludin and is essential for male fertility (Saitou et al., 2000). *Ocln* showed the highest expression during stages IX-XII (Fig. 5A), and this expression was increased by RA treatment in cultured Sertoli cells in a similar manner to other stage-dependent genes showing an expression peak during stages VII-XII (Fig. 5B). Although occludin was detectable throughout the spermatogenic cycle, as previously reported (Cyr et al., 1999), the amount was reduced during stages VII-VIII in normal spermatogenesis (supplementary material Fig. S9), which might be a result of post-translational regulation of occludin (Xia et al., 2009; Su et al., 2010), and the reduction occurred normally even in testes injected with LV-dnRARα. However, occludin expression remained at a low level even in stage IX-XII tubules at 5 days after injection of LV-dnRARα but not at the other stages (Fig. 5C-F). We conclude that the continuous decrease of occludin at stages IX-XII is caused by the decline of de novo synthesis of occludin from stage IX owing to reduced mRNA.

Although occludin KO mice showed massive germ cell loss (Saitou et al., 2000), the phenotype of the BTB and the cause of germ cell loss in these mutant mice have remained unclear. We therefore attempted to knock down occludin in our present study using lentivirus-mediated in vivo RNAi and thereby determine whether this protein is involved in BTB organization and is causative for the defects induced by overexpression of dnRARα (supplementary material Fig. S10; Fig. 6). We prepared two lentiviral constructs containing shRNAs for occludin (LV-Ocln-i1, Ocln-i2), efficiencies of which were found to be ~60% and ~80% reduction in primary Sertoli cells, respectively (supplementary material Fig. S10B). We then injected LV-Ocln-
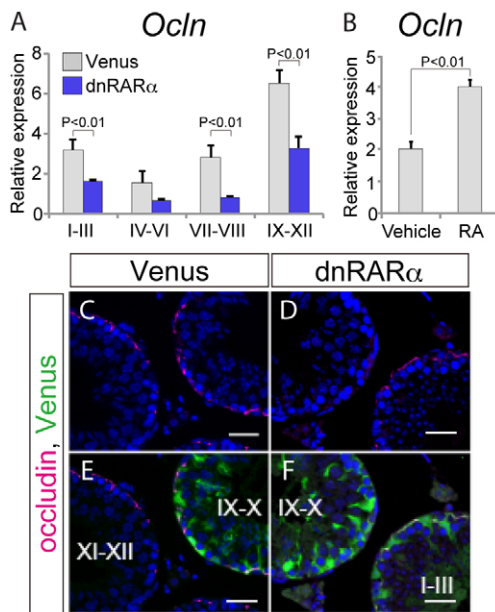
Fig. 5. Reduced expression of occludin in LV-dnRARα-injected tubules. (**A**) LV-dnRARα or LV-Venus were injected into mouse testes, and the stage-dependent expression of *Ocln* in isolated tubules was quantified by qRT-PCR (*n*=3). (**B**) Cultured Sertoli cells were treated with 1 µM RA for 24 hours and expression changes in *Ocln* were quantified by qRT-PCR (*n*=3). *Gapdh* was used as an internal control. (**C-F**) Immunostaining for occludin (magenta) and Venus (green) on sections of testes injected with LV-Venus or LV-dnRARα. Error bars represent s.d. Scale bars: 40 µm.

i1 or i2 into adult testes, and examined occludin expression and BTB organization. At day 5 post-injection, *Ocln* mRNA was decreased at all stages, as expected (supplementary material Fig. S10C). Interestingly, however, immunostaining revealed that occludin expression decreased only at stages IX-XII but not at the other stages, similar to the findings in the LV-dnRARα-injected testes (Fig. 6A-D). Moreover, the permeability of the BTB was affected in stage IX-XII tubules (Fig. 6E-H,Q), which is consistent with the observation that ZO1 expression was reduced only in stage IX-XII tubules (Fig. 6I-L). These results indicate that de novo synthesis of occludin is required for BTB organization from stage IX.

We evaluated next whether occludin plays any role in stage-specific germ cell survival. TUNEL assay results revealed that apoptosis was elevated in the spermatocytes and spermatids of occludin knockdown mice during stages IX-XII, but not at other stages (Fig. 6M-P,R). These results suggested that occludin is important for germ cell survival possibly via a role in BTB organization. However, other abnormalities observed in LV-EF-dnRARα-injected testes, such as the reduced expression of ZO1 at stages VII-VIII, the mislocalization of elongated spermatids, the degeneration of the seminiferous epithelium and an abnormal localization of Sertoli cells, were not found to be induced by the knockdown of occludin (supplementary material Fig. S10D-G). Taken together, these data indicate that the reduction of occludin expression is responsible, at least in part, for the disorganization of the BTB and the apoptosis of differentiating germ cells in stage IX-XII tubules, which are induced also by the overexpression of dnRARα in Sertoli cells.

DISCUSSION

We find in our present study that the overexpression of dnRARα in Sertoli cells leads to abnormal BTB regulation during stages VII-XII and this is partially due to reduction of occludin expression. We have also found that RA signaling plays an important role in the regulation of stage-dependent gene expression in Sertoli cells, including that of *Ocln*. We propose that the periodic activation of RA signaling in Sertoli cells contributes to a stage-dependent regulation of the BTB, as shown in Fig. 7.

Stage-dependent regulation of BTB and other junctional components in Sertoli cells by RA signaling

The BTB was disrupted during stages VII-XII at day 5 after injection of LV-dnRARα. Because it has been suggested that old and new TJs are disassembled and reassembled around stages VII and VIII, respectively, the activation of RA signaling might change the transcriptional status in Sertoli cells and induce the reorganization of TJs, especially through de novo synthesis of TJ-related proteins. The degeneration of seminiferous tubules and apoptosis of germ cells in the testes overexpressing dnRARα would be mainly caused by dysfunction of the BTB. We identified occludin as a tight junction protein partially responsible for the abnormalities induced by overexpression of dnRARα at stages IX-XII. Although their mRNAs were reduced throughout the cycle, reduction of the proteins was observed only at those stages, indicating the importance of the stage-specific induction of occludin expression by RA signaling. As short-term suppression of *Ocln* expression did not affect the amount of occludin or the permeability of BTB during stages VII-VIII, the proteins detectable at those stages might be undergoing degradation (Xia et al., 2009; Su et al., 2010) and be dispensable for the organization of new TJs. The abnormalities we observed at day 5 after the knockdown of occludin were milder than those induced by overexpression of dnRARα and were restricted to stages IX-XII. These milder phenotypes could be explained by the dysfunction of other genes affected by overexpression of dnRARα.

RA signaling might also regulate the stage-dependent function of other junctional components in Sertoli cells. The evidence for this includes the presence of potential RA responsive element sites in the promoters of genes encoding components of gap junctions, and the dysfunctional gap junction found in the RARα knockout (KO) mouse (Chung et al., 2010). Furthermore, vimentin, an intermediate filament protein tightly associated with the localization of elongated spermatids, has also been reported to be disrupted in RARα KO mice (Chung et al., 2010). Accordingly, the abnormal transition and release of elongated spermatids might be caused by the misregulation of intermediate filaments. Taken together, our current data and previous findings indicate that the cyclical activation of RA signaling in Sertoli cells is likely to orchestrate the stage-dependent expression and function of genes involved in TJs and other junctional components.

Regulation of stage-dependent gene expression in Sertoli cells by RA signaling

The cyclicity of spermatogenesis has been studied for several decades (Clermont, 1972; Hogarth and Griswold, 2010), but its regulation has remained elusive. In our current study, we find that gene expression in mouse Sertoli cells is divided into two phases, depending on the properties of the genes in relation to their response to RA. During stages I-VI, the genes suppressed by RA signaling show peak expression whereas the genes activated by RA signaling
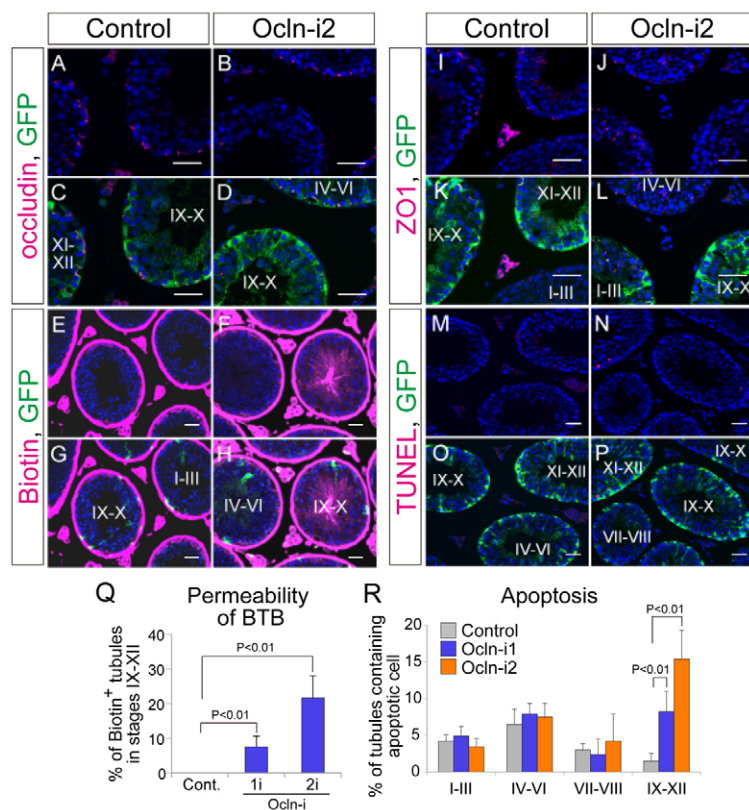
Fig. 6. Disruption of BTB organization and stage-specific germ cell apoptosis induced by knockdown of occludin. (**A-D**) Immunostaining for occludin (magenta) and GFP (green) on sections from mouse testes injected with LV-control or LV-Ocln-i2. Stages were determined by staining of serial sections with PAS and Hematoxylin. (**E-H**) Functional assessment of BTB. Distribution of biotin tracer (magenta) was visualized with streptavidin conjugated with Alexa488, and GFP (green) was detected with a GFP antibody. (**I-L**) Immunostaining for ZO1 (magenta) and GFP (green) on sections of testes injected with LV-control or LV-Ocln-2i. (**M-P**) TUNEL staining (magenta) and immunostaining for GFP (green) in testes injected with LV-control or LV-Ocln-2i. (**Q**) Quantification of tubules showing biotin permeability in GFP-positive tubules at stages IX-XII. Only GFP-positive tubules were counted (*n*=4). (**R**) Quantification of stage-dependent apoptosis in testes injected with LV-Ocln-i. Only GFP-positive tubules were counted (*n*=3). Error bars represent s.d. Scale bars: 40 μm.

show peak expression during stages VII-XII. Hence, our findings suggest that RA signaling is periodically activated at stages VII-XII and induces stage-dependent gene expression changes in Sertoli cells (supplementary material Fig. S8). By perceiving the local concentration of RA, Sertoli cells would create a cyclical microenvironment essential for the development of stage-specific germ cells and then maintain this process continuously. Spermatogenesis during stages I-VI did not show any evidence of abnormalities at day 5 after overexpression of dnRARα. To examine in the future whether lower activation of RA signaling in Sertoli cells during stages I-VI has any role in spermatogenesis, a system that enables hyperactivation of RA signaling will be required.

Although genes involved in RA metabolism are expressed in both germ cells and somatic cells (Vernet et al., 2006b), germ cells might regulate the local metabolism of RA. It has been suggested that germ cells control the spermatogenic cycle, as evidence has been presented showing that when rat germ cells are transplanted into a mouse testis, spermatogenesis proceeds with a cycle that is characteristic of the rat (França et al., 1998). Furthermore, Sertoli cell-specific RARα CKO mice show disruption of stage-dependent gene expression but the seminiferous epithelial stages remain intact (Vernet et al., 2006a). Recently, it was reported that late pachytene spermatocytes and round spermatids might control local metabolism of RA and orchestrate differentiation of spermatogonia and reset the Sertoli cell cycle to stage VII (Sugimoto et al., 2012). Accordingly, the differentiating germ cells may transmit information regarding their developmental stage to the Sertoli cells via the abundance of RA and the Sertoli cells might then change the microenvironment so that it is suitable for the germ cells. This kind of communication feedback between somatic and spermatogenic cells might be important for the continuous development of spermatogenic cells.

In our current analysis, we identified a total of 419 stage-dependent genes in Sertoli cells by microarray and found that ~40% of these genes respond to RA signaling in vivo. However, other signaling pathway(s) are likely to be involved in the cyclic regulation of Sertoli cells and spermatogenesis as our comprehensive study has revealed that stage-dependent gene expression can be classified into more than four types. In this regard, RA signaling divides the cycle into only two types and therefore cannot create the entire stage-dependent gene expression in Sertoli cells. In addition, the expression of many genes remains unchanged even after overexpression of dnRARα. *Notch1* receptor and its downstream target *Hes1* have been reported to show stage-dependent gene expression (Hasegawa et al., 2012), but these factors did not exhibit expression changes upon overexpression of dnRARα (supplementary material Table S2). This finding indicates that RA signaling and Notch signaling in Sertoli cells might be independently regulated. Our current microarray data include some components of other signaling pathways, such as Egf, Fgf, Vegf and Pdgf, which might also be involved in the cyclicity of Sertoli cells and spermatogenesis.

Different phenotypes induced by overexpression of dnRARα and Sertoli cell-specific deletion of RARα

The phenotypes observed in mouse testes injected with LV-dnRARα in our present study were more severe than those observed previously in Sertoli cell-specific RARα CKO mice (Vernet et al., 2006a). We suggest that this discrepancy could be ascribed to the different effects of dominant-negative suppression and the deletion of RARα. It is believed that RARs generally bind to DNA together with co-regulators, even in the absence of RA, and alter the transcriptional status by exchanging co-regulators after
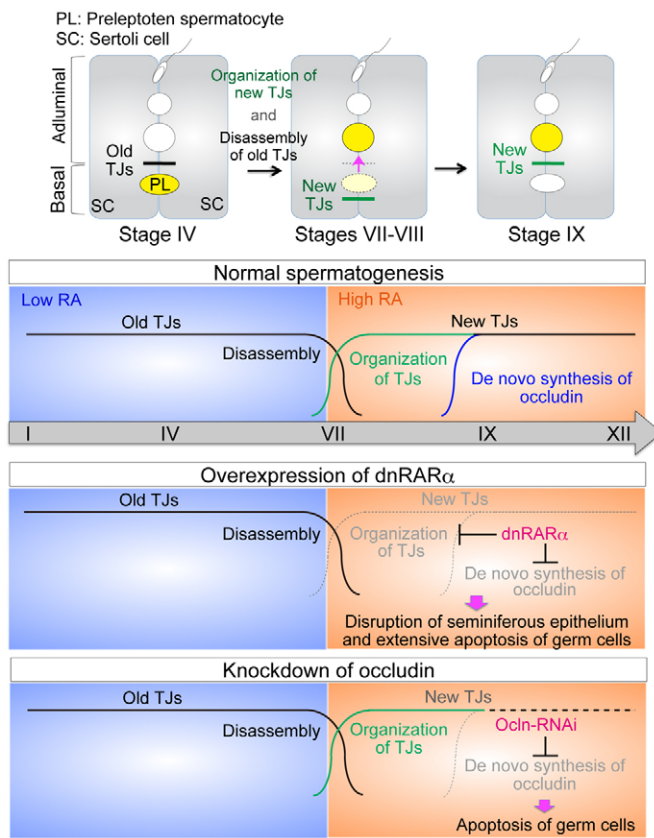
Fig. 7. Proposed model for a periodic organization of TJs by RA signaling. During normal spermatogenesis, the disassembly of old TJs occurs to ensure the translocation of preleptotene spermatocytes into the adluminal compartment around stages VII-VIII. Concomitantly, new TJs are reorganized to maintain BTB integrity. New occludin proteins are also synthesized and incorporated into new TJs by the end of stage VIII. The activation of RA signaling from stage VII would be required for the reorganization of new TJs and also the de novo synthesis of occludin. Hence, the overexpression of dnRARα may impair the formation of new TJs from stage VII, which leads to the severe disruption of the seminiferous epithelium and extensive apoptosis of germ cells. RNAi targeting of *Ocln* suppresses the de novo synthesis of occludin proteins, which may lead to defective TJ organization and cause the mild apoptosis of germ cells from stage IX.

RA binding (Rosenfeld et al., 2006). Hence, dnRARα may bind to DNA and continue to suppress or activate the transcription of target genes and never exchange co-regulators even in the presence of RA. By contrast, it was reported that in RARα CKO mice, the stage-dependent gene expression change is abolished but the total expression level of stage-dependent genes in the mutant testes did not differ from those in wild type (Vernet et al., 2006a). Thus, it is expected that both ligand-dependent and -independent activation or repression by RARα might be diminished in the RARα CKO mice (supplementary material Fig. S8). Such differences might generate distinct transcriptional states for target genes controlled by RARα, which may lead to the manifestation of different phenotypes. A second possibility is that dnRARα induces the severe phenotype through a non-canonical pathway of RA signaling (Andersen and Rosenfeld, 1995; Chen and Lohnes, 2005). It was reported that overexpression of dnRARα in basal keratinocytes resulted in an

epidermal defect that was more severe than that observed in RARαγ double-null mutant mice, and that the overexpression of dnRARα^{DBD}, which is incapable of DNA binding, recapitulated the epidermal phenotype induced by dnRARα. This report suggests that dnRARα might impact on gene expression in Sertoli cells through a DNA-receptor independent manner. Although we cannot rule out this possibility, many of the stage-dependent genes, including *Ocln*, responded to RA treatment. Therefore, our results suggest that RA signaling is, at least in part, involved in a stage-dependent organization of the BTB. Another possibility is that the different phenotypes might be due to the genetic background. In our present experiments, we used C57BL6/j mice and knockout mice of this background often show a more severe phenotype than those of other strains (Lin and Page, 2005; Anderson et al., 2008; Mark et al., 2008).

Conclusions

Our current findings indicate that the stage-dependent activation of RA signaling coordinates gene expression in Sertoli cells and act as a fundamental regulator of BTB organization. These data expand the current mechanistic understanding of the cyclicity of spermatogenesis and also provide a foundation for further elucidation of the signaling pathway(s) regulating the cycle of Sertoli cells and spermatogenesis.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080119/-/DC1

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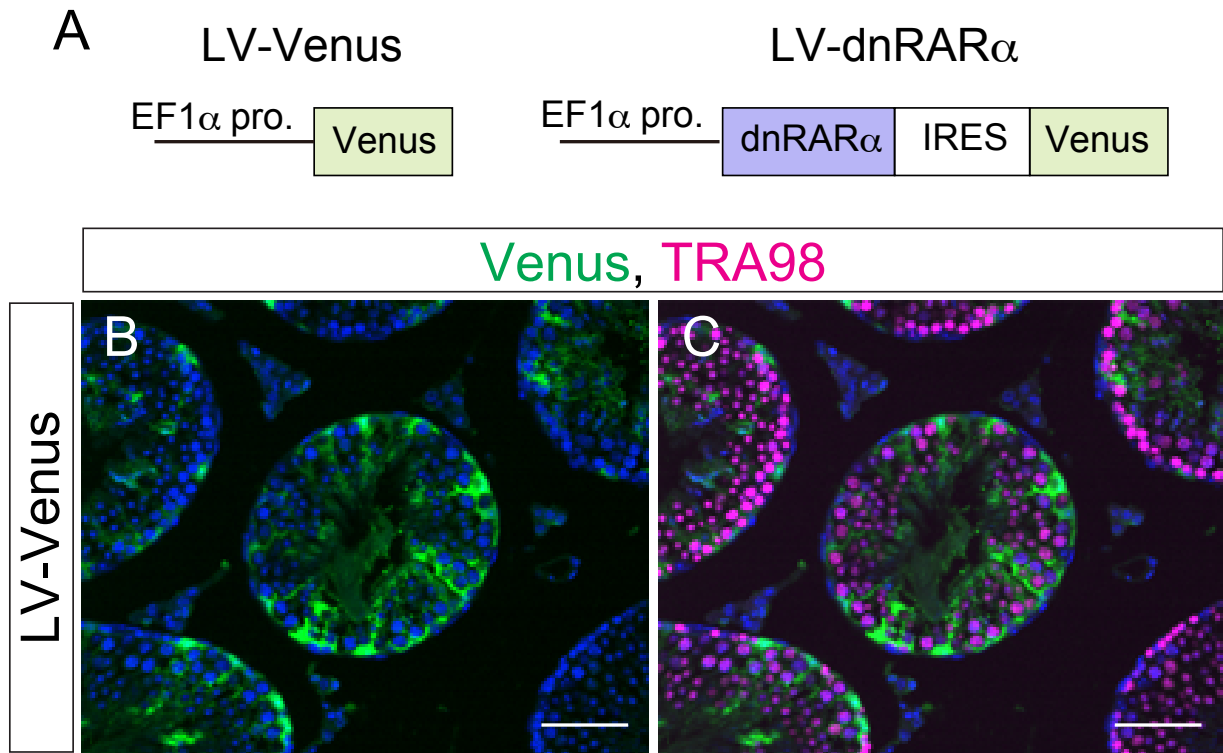
Fig. S1. Confirmation of cell type-specific infection of lentivirus. (**A**) Construction of LV-Venus and LV-dnRARα vectors. (**B,C**) At day 5 after the injection of LV-Venus, expression of broad germ cell marker TRA98 (magenta) and Venus (Green) were examined with immunohistochemistry. Scale bars: 80 μm.
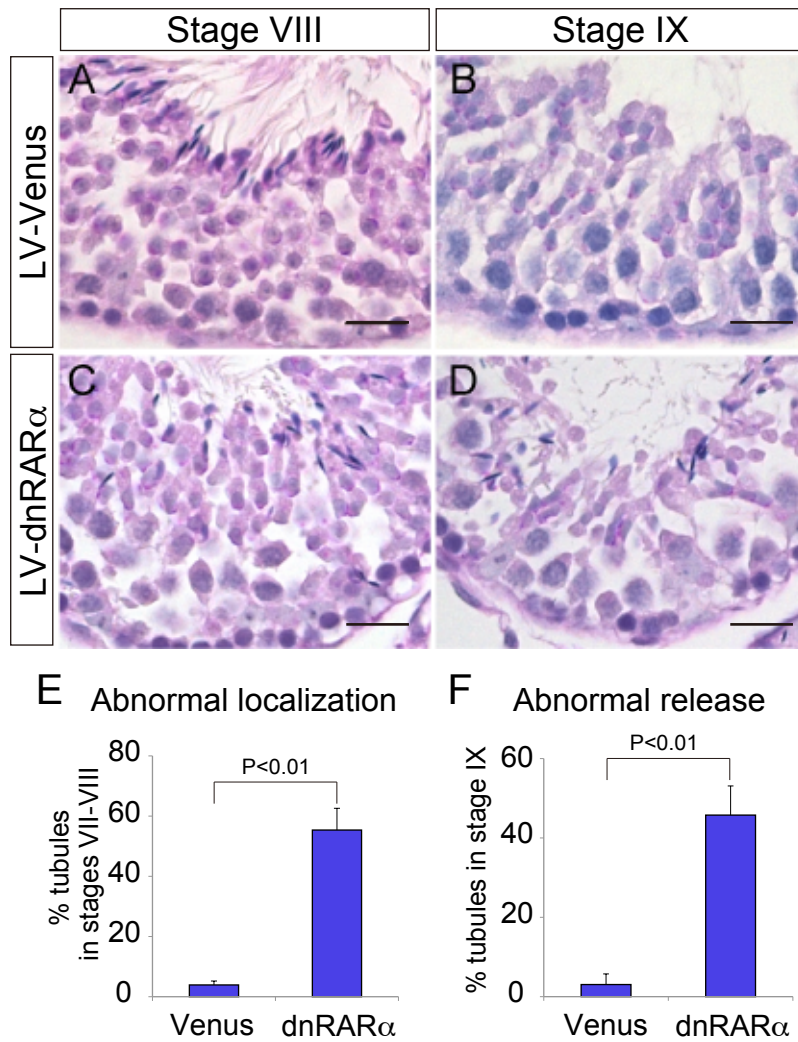
Fig. S2. Abnormal transition and release of elongated spermatids upon the overexpression of dnRARα. (A-D) Histological sections derived from mouse testes injected with LV-Venus or LV-dnRARα at stage VIII or stage IX. (**E**) Proportion of Venus-positive tubules at stages VII-VIII showing abnormal alignment of elongated spermatids (*n*=4). (**F**) Proportion of Venus-positive tubules at stage IX containing abnormally retained elongated spermatids (*n*=4). Only Venus-positive tubules were counted. Error bars, s.d. Scale bars: 20 μm.
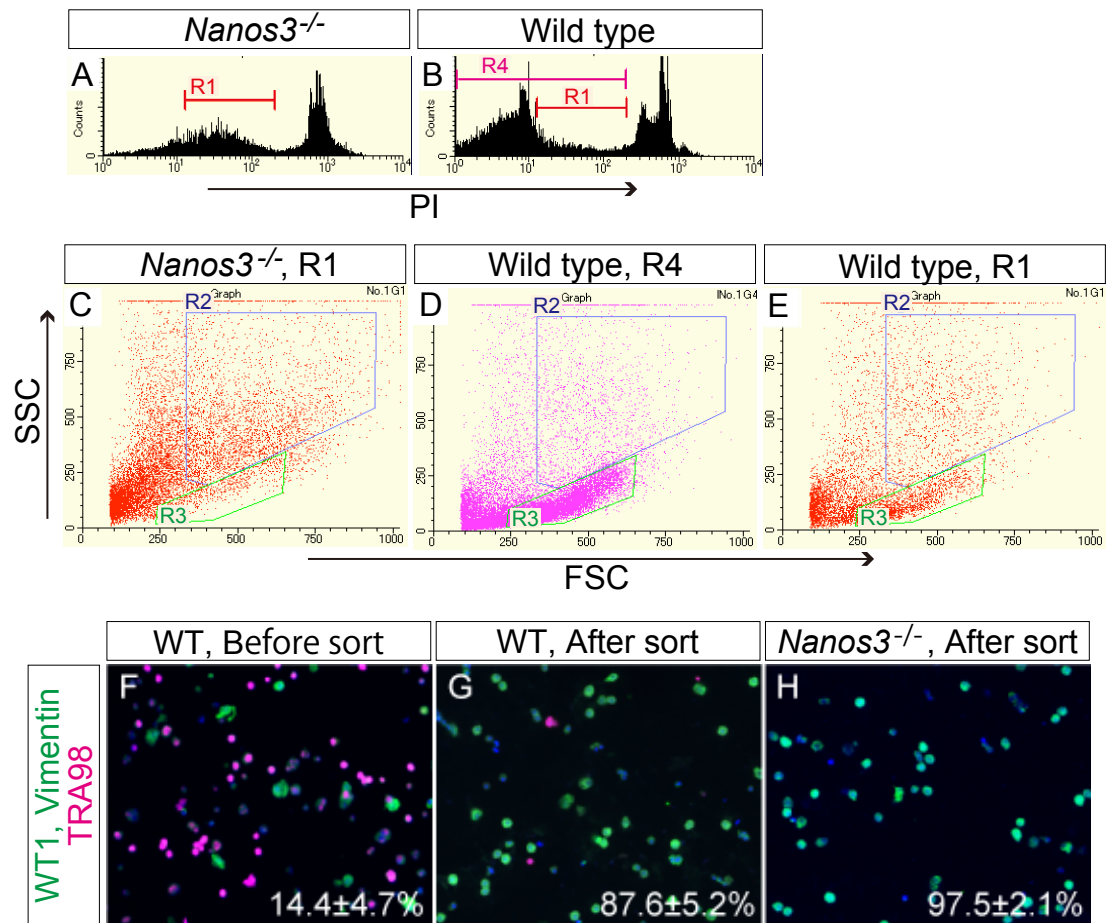
Fig. S3. Direct isolation of Sertoli cells from adult testes by FACS. (**A**,**B**) Testicular cells obtained from *Nanos3*⁻ᐟ⁻ or wild-type mice were analyzed by FACS. Propidium iodide (PI)-negative cells (R4) or cells showing high intrinsic fluorescence (R1) were selected for flow sorting. PI-positive dead cells were removed. (**C-E**) R1 fractions from *Nanos3*⁻ᐟ⁻ or wild-type testicular cells and an R4 fraction from wild-type testicular cells. R2 and R3 fractions represent Sertoli cells and germ cells, respectively. (**F-H**) Wild-type testicular cells before and after sorting and *Nanos3*⁻ᐟ⁻ testicular cells after sorting were immunostained with antibodies against WT1, vimentin (green) and TRA98 (magenta). The proportions of WT1 and vimentin-positive Sertoli cells are indicated at the bottom-right of the lower panels. The values are the mean ± s.d.
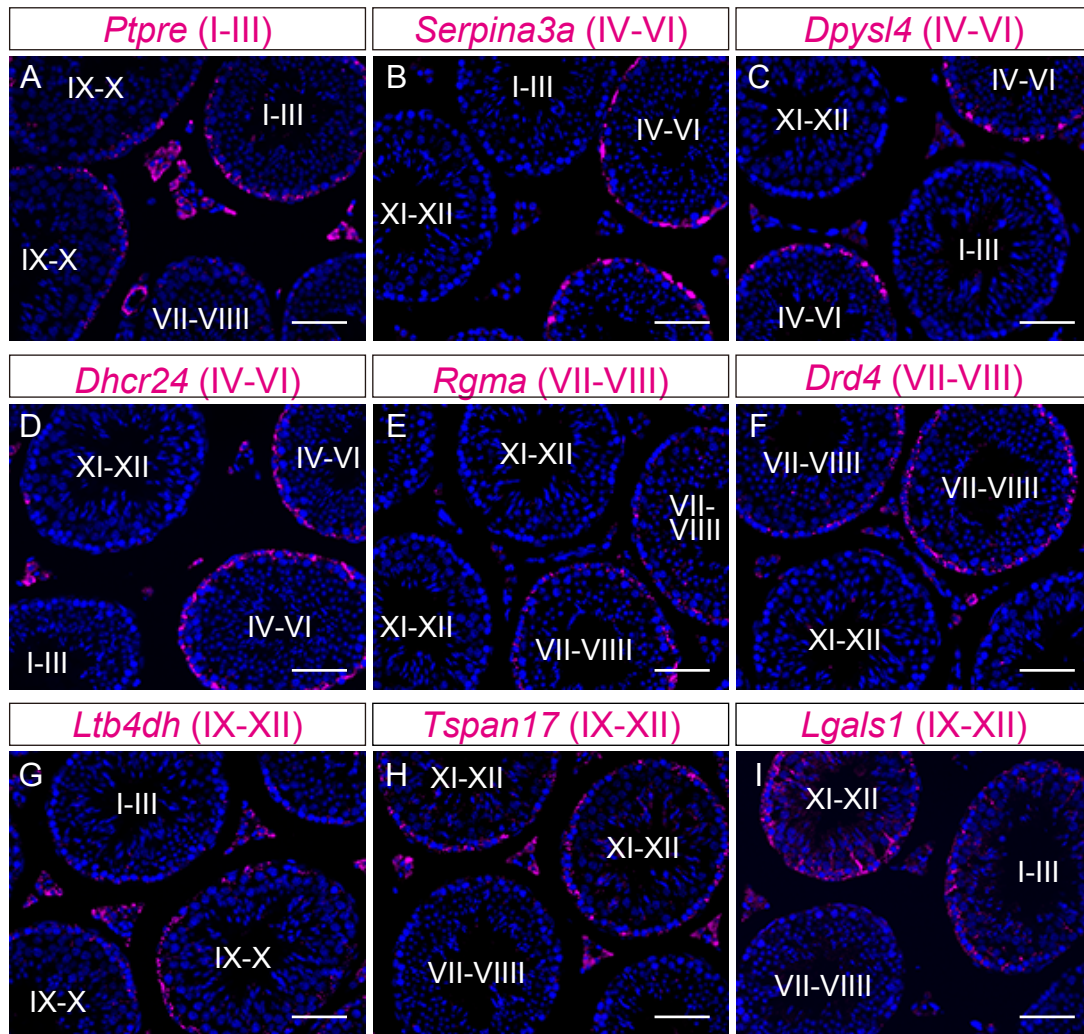
Fig. S4. Validation of the microarray results. (**A-I**) The spatial expression patterns of nine genes identified as stage-dependent and showing peaks during stages I-III (*Ptpre*), IV-VI (*Serpina3a*, *Dpysl4*, *Dhcr24*), VII-VIII (*Rgma*, *Drd4*) and IX-XII (*Ltb4dh, Tspan17*, *Lgals1*) were examined by in situ hybridization. The seminiferous epithelial stages were determined by serial section staining with PAS and Hematoxylin. Scale bars: 80 μm.
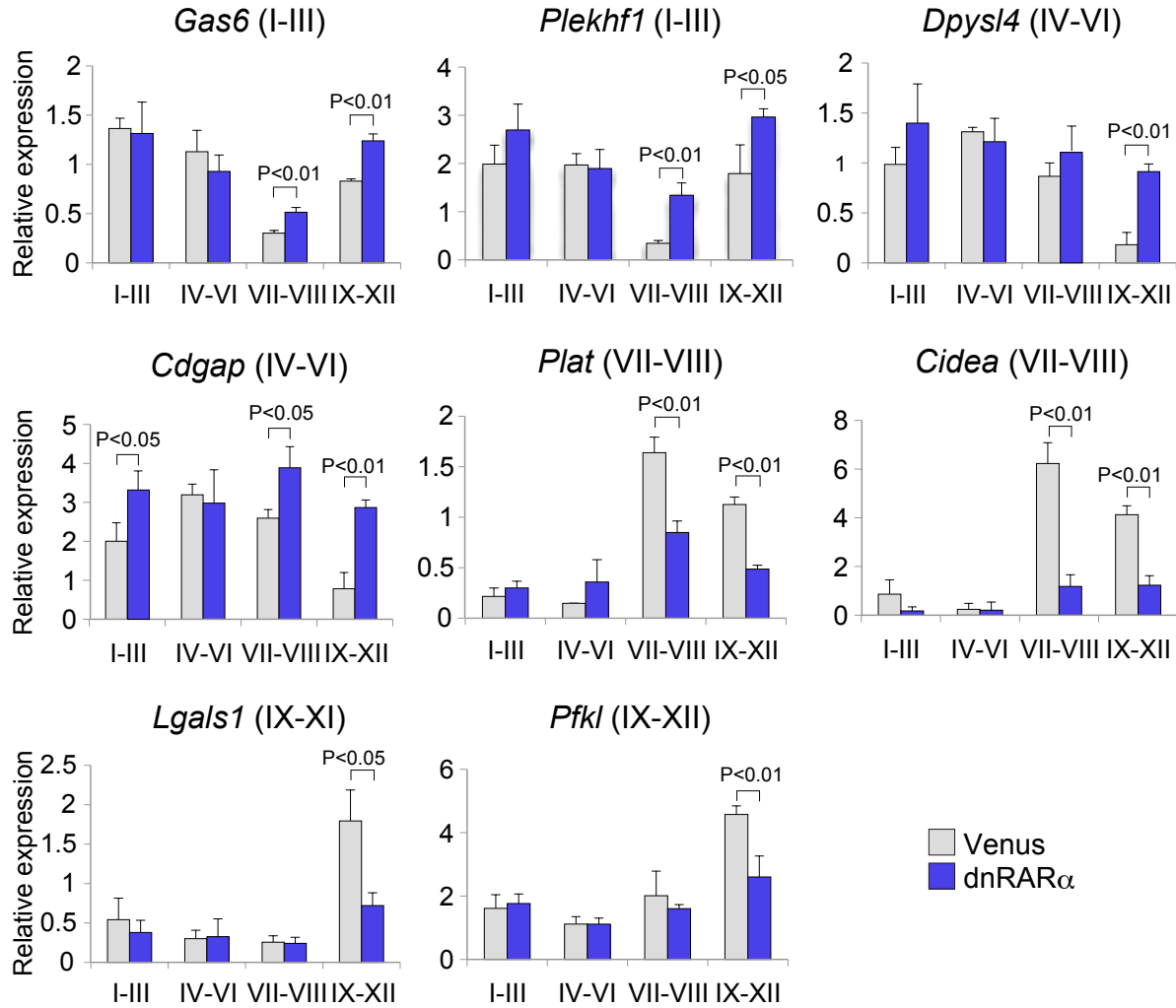
Fig. S5. Validation of the gene expression changes induced by the overexpression of dnRARα. At day 5 after the injection of LV-dnRARα or LV-Venus, stage-specific Venus⁺ tubules were isolated and the expression of the indicated stage-dependent genes showing peaks during stages I-III (*Gas6*, *Plekhf1*), IV-VI (*Dpysl4*, *Cdgap*), VII-VIII (*Plat*, *Cidea*) and IX-XII (*LgalsI*, *Pfkl*) were quantified by qRT-PCR (*n*=3). *Gapdh* was used as internal control. Error bars, s.d.
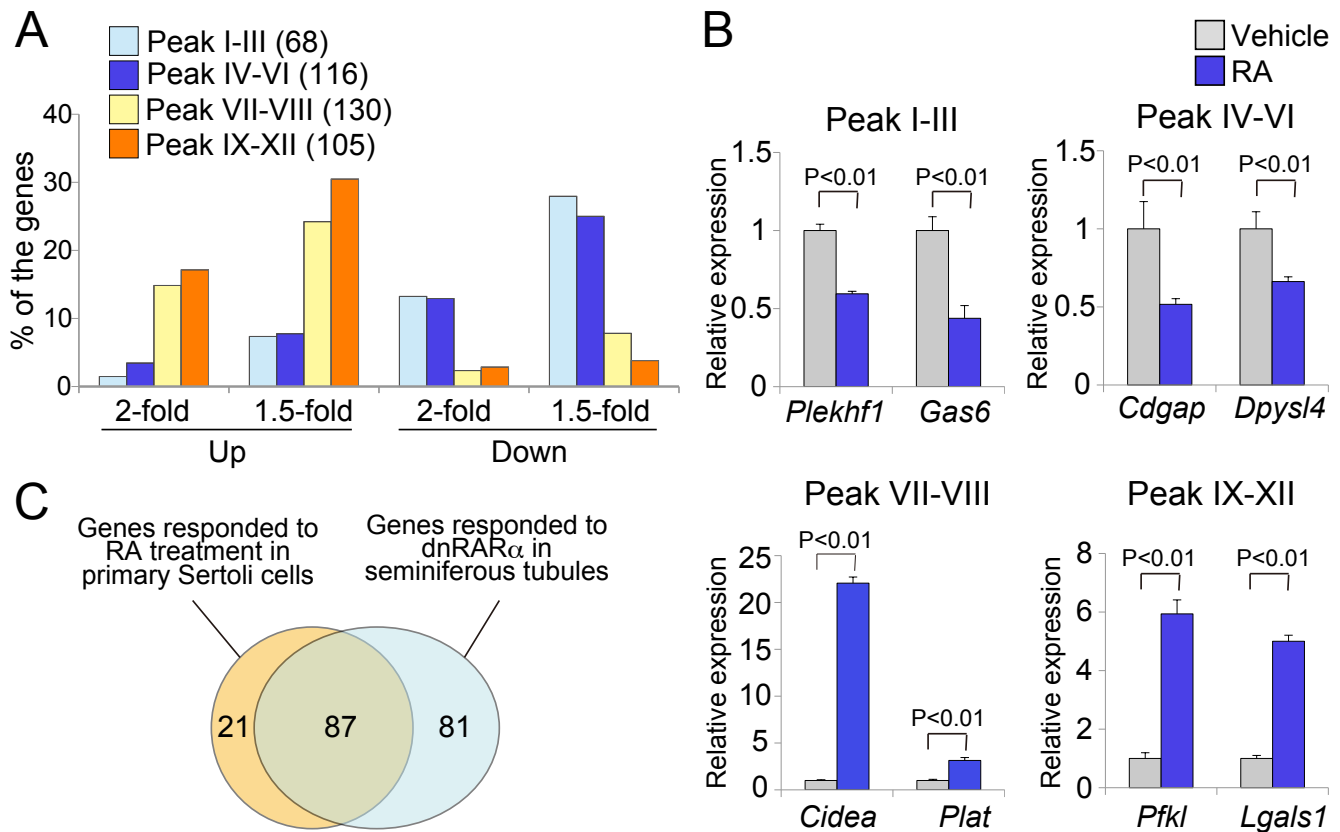
Fig. S6. Expression changes of the stage-dependent genes upon the activation of RA signaling in primary Sertoli cells. (**A**) Cultured Sertoli cells were incubated with 1 μM RA for 24 hours and the expression changes of stage-dependent genes were measured by microarray (*n*=2). *y*-axis represents total percentage of genes that showed up- or downregulation. (**C**) Comparison of genes that responded to RA treatment in primary Sertoli cells (108 genes) and to overexpression of dnRARa in seminifero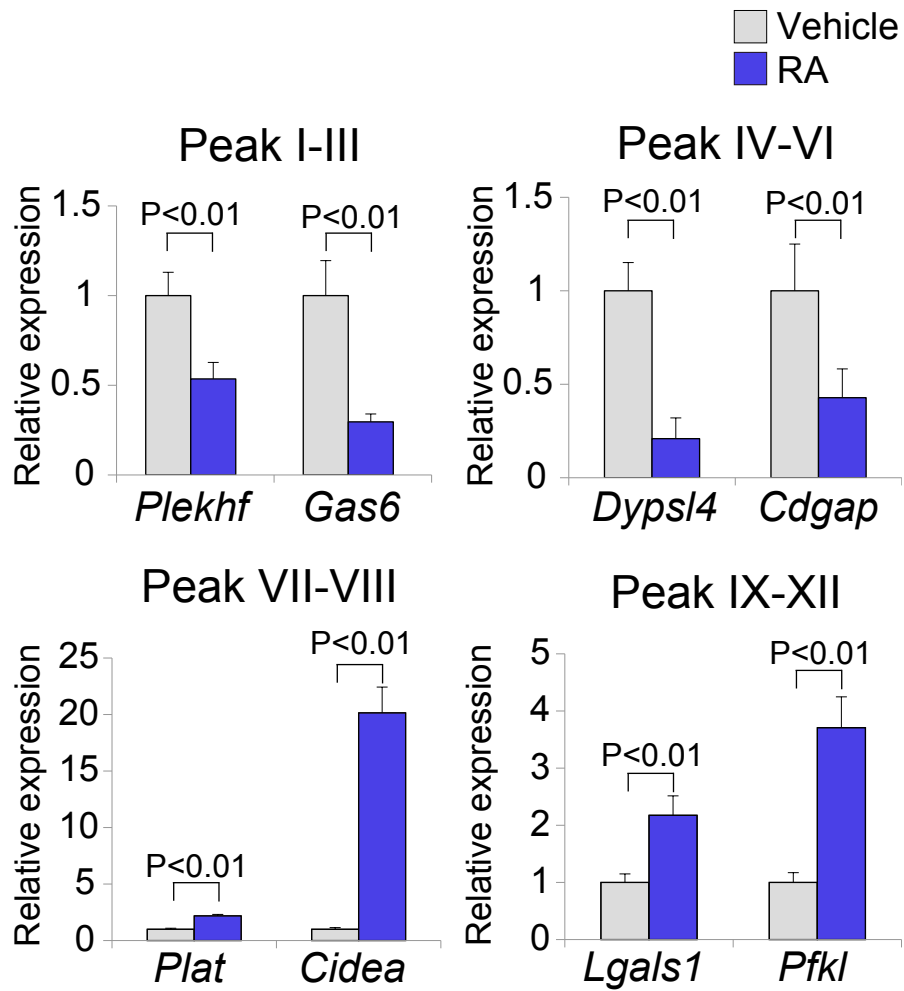us tubules (168 genes). Between those, 87 genes were common. (**B**) To validate the microarray results, the expression of stage-dependent genes showing peaks during stages I-III *(Plekhf, Gas6)*, IV-VI (*Cdgap*, *Dpysl4*), VII-VIII (*Cidea*, *Plat*) and IX-XII (*Pfkl*, *Lgals1*) in cultured Sertoli cells treated with RA were quantified using qRT-PCR (*n*=3). *Gapdh* was used as internal control. Error bars, s.d.

Fig. S7. Expression changes of stage-dependent genes in VAD mice following retinol injections. At 24 hours post-injection, genes showing peak expression during stages I-III (*Plekhf1*, *Gas6*), IV-VI (*Dpysl4*, *Cdgap*), VII-VIII (*Plat*, *Cide*a) and IX-XII (*Lgals1*, *Pfkl*) in whole mouse testes were assayed by qRT-PCR (*n*=3). *Gapdh* was used as internal control. Error bars, s.d.
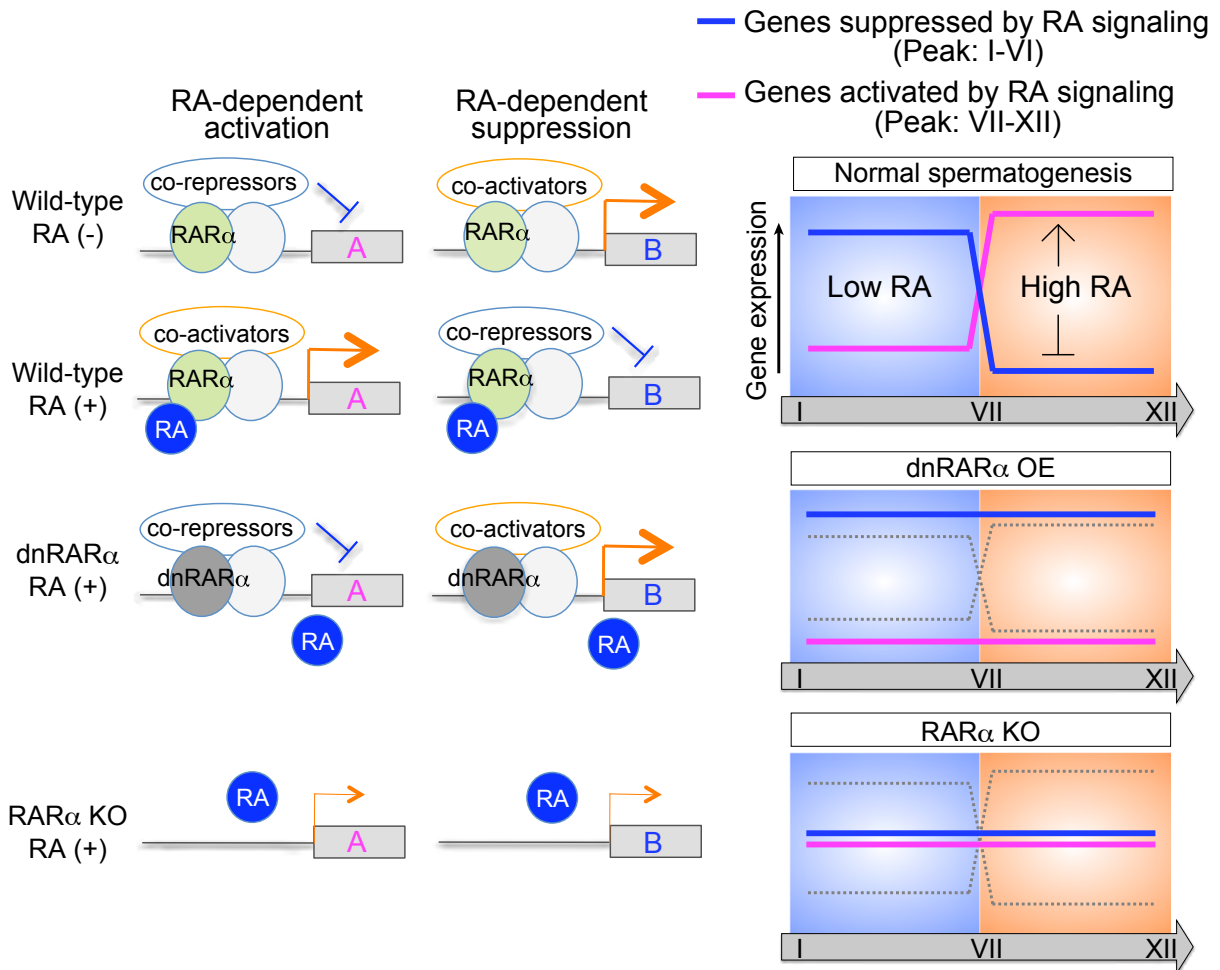
Fig. S8. Proposed model for regulation of stage-dependent gene expression in Sertoli cells by RA signaling. RARα usually binds to DNA and activates or suppresses the corresponding target genes, even in the absence of RA. Upon the binding of RA, RARα exchanges the co-regulators and alters the transcription status of the target genes. During normal spermatogenesis, RA signaling is maintained at low and high levels during stages I-VI and VII-XII, respectively, and thereby creates two patterns of gene expression in Sertoli cells through the regulation of RA-responsive genes. Genes suppressed and activated after the binding of RA to RARα show expression peaks during stages I-VI and VII-XII, respectively. In the case of the overexpression (OE) of dnRARα, the exchange of co-regulators would not occur even in the presence of RA. As a result, the genes controlled by RA signaling will remain in a stage I-VI-like expression state. In the case of a RARα KO, both RA-dependent and -independent activation or suppression might be diminished.
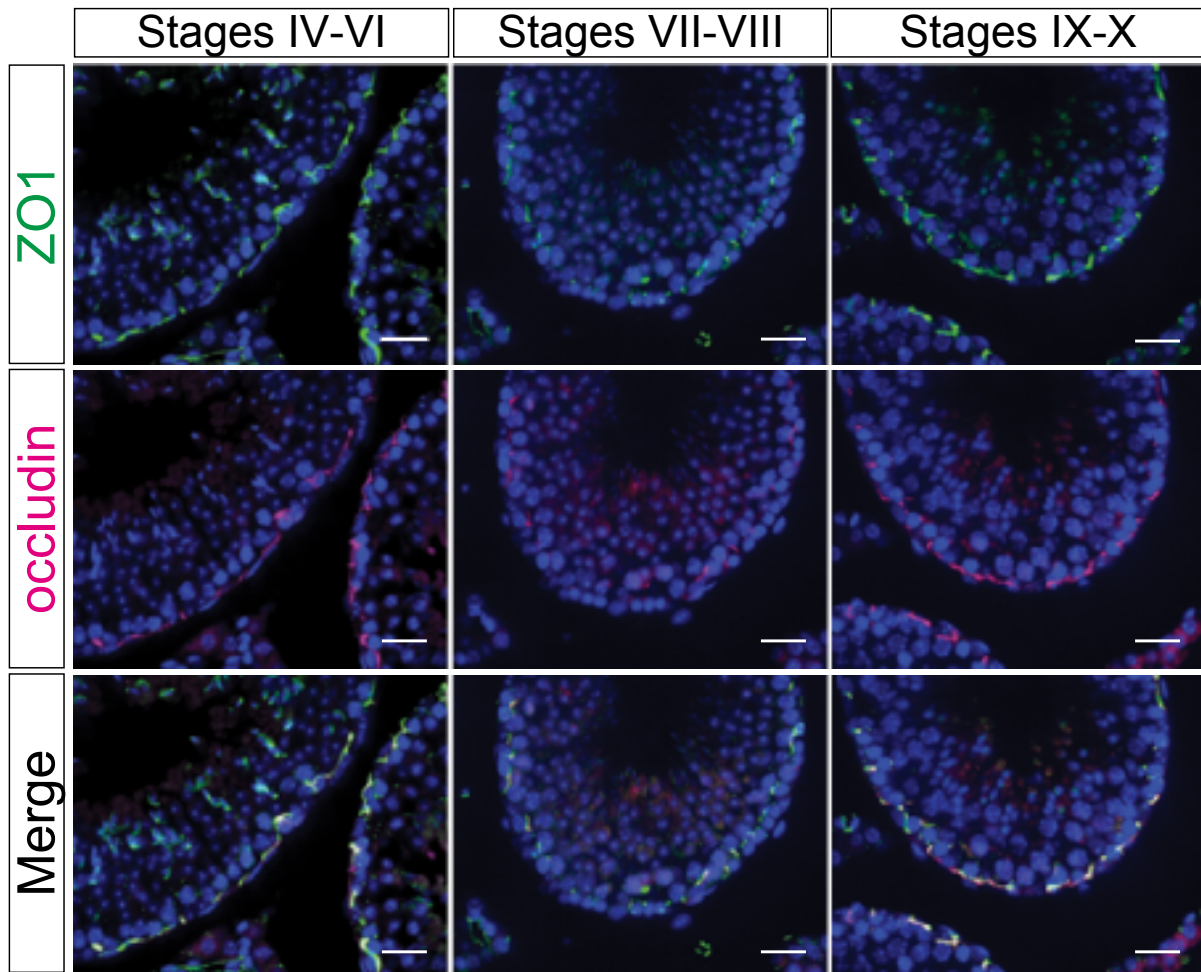
Fig. S9. Stage-dependent expression change of occludin. Immunostaining for ZO1 (green) and occludin (magenta) in stage-specific tubules. Seminiferous epithelial stages were determined with serial sections with PAS and Hematoxylin. Scale bars: 20 μm.
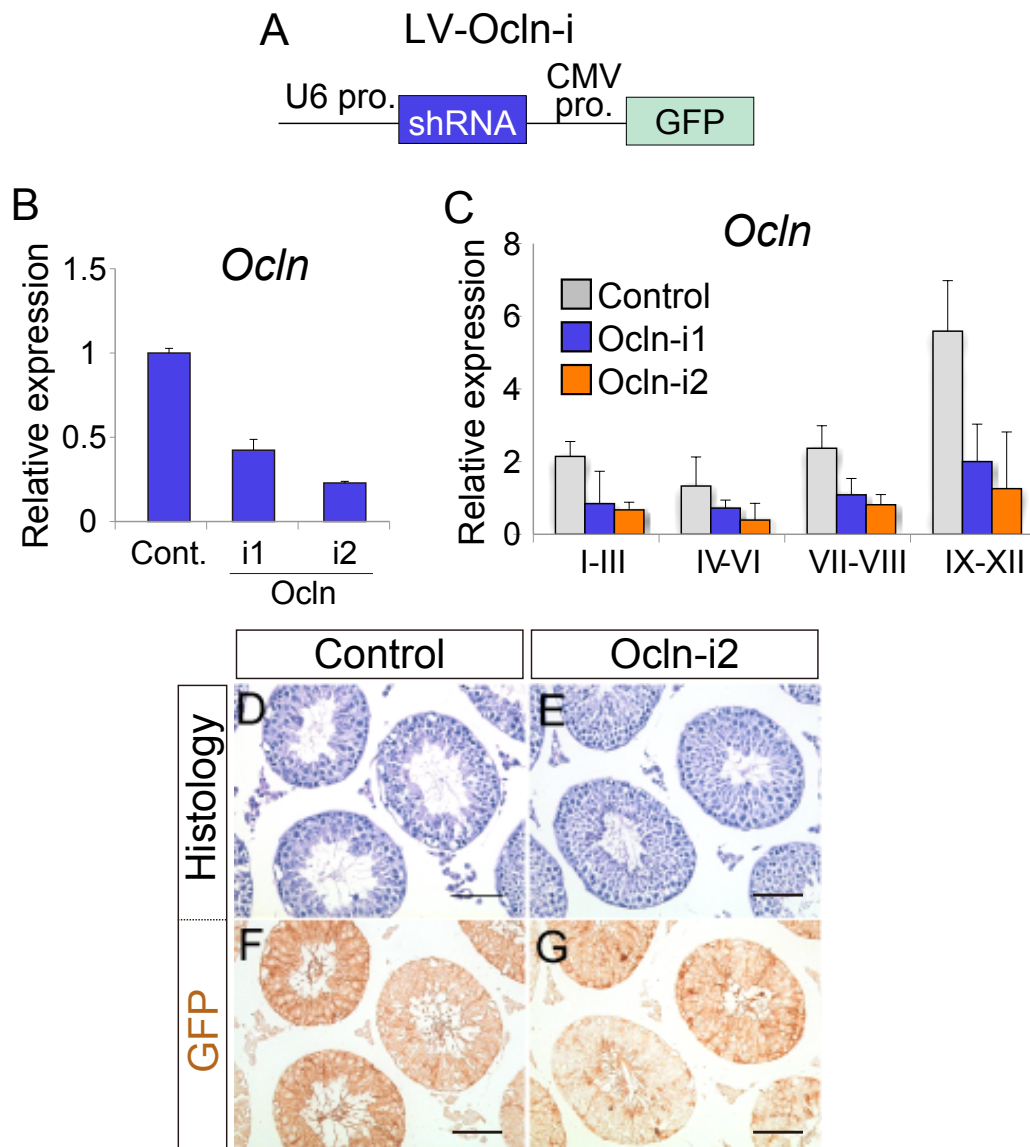
Fig. S10. Efficiency of RNAi in suppressing *Ocln* expression. (**A**) Construction of the LV-Ocln-i vector. Cells infected with this lentiviral construct start to express both shRNA from the U6 promoter and GFP from the CMV promoter. (**B**) Cultured Sertoli cells were infected with LV-Ocln-i1 or -i2 and the expression of *Ocln* was subsequently quantified by qRT-PCR (*n*=3). (**C**) Adult mouse testes were injected with LV-Ocln-i1 or i2 and changes in the stage-dependent expression of *Ocln* were then quantified by qRT-PCR (*n*=3). *Gapdh* was used as internal control. (**D-G**) Histological analysis of mouse testes at two weeks after injection of LV-control or LV-Ocln-i2 vectors. Tissue sections were stained with PAS and Hematoxylin. Error bars, s.d. Scale bars: 40 μm.