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Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells

Nadège Vernet[1], Christine Dennefeld[1], Florian Guillou[2], Pierre Chambon[1], Norbert B Ghyselinck[1,*] and Manuel Mark[1,*]

[1]IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM, U596, Illkirch, France; CNRS, UMR7104, Illkirch, France; Faculté de Médecine, Strasbourg, France and [2]INRA, Université de Tours, Nouzilly, France

Sertoli cells (SC) are instrumental to stem spermatogonia differentiation, a process that critically depends on retinoic acid (RA). We show here that selective ablation of RA receptor alpha (RARalpha) gene in mouse SC, singly (*Rara*$^{Ser-/-}$ mutation) or in combination with RARbeta and RARgamma genes (*Rara/b/g*$^{Ser-/-}$ mutation), abolishes cyclical gene expression in these cells. It additionally induces testis degeneration and delays spermatogonial expression of *Stra8*, two hallmarks of RA deficiency. As identical defects are generated upon inactivation of RARalpha in the whole organism, our data demonstrate that all the functions exerted by RARalpha in male reproduction are Sertoli cell-autonomous. They further indicate that RARalpha is a master regulator of the cyclical activity of SC and controls paracrine pathways required for spermatogonia differentiation and germ cell survival. Most importantly, we show that the ablation of all RXR (alpha, beta and gamma isotypes) in SC does not recapitulate the phenotype generated upon ablation of all three RARs, thereby providing the first evidence that RARs exert functions *in vivo* independently of RXRs.

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Introduction

The mammalian seminiferous epithelium consists of Sertoli cells (SC) and germ cells. Its development, renewal and functioning, which underlie spermatogenesis, require a complex assortment of hormones and cytokines (Meng *et al*, 2000; Holdcraft and Braun, 2004). Among these signals, retinoic acid (RA), the active metabolite of vitamin A (retinol), regulates spermatogonia differentiation and spermatid adhesion properties (Ghyselinck *et al*, 2006; Vernet *et al*, 2006). RA acts through binding to nuclear retinoic acid receptors (RARα, β and γ isotypes), which are ligand-dependent transcriptional regulators transducing the RA signal in the form of heterodimers with the rexinoid receptors, RXRα, β and γ (Kastner *et al*, 1997; Chambon, 2005; Mark *et al*, 2006). During post-natal development and adulthood, each RAR is detected predominantly in a specific cell type of the seminiferous epithelium: RARα in SC, RARβ in round spermatids and RARγ in A spermatogonia (Vernet *et al*, 2006). Germline inactivation of *Rara* results in testis degeneration comprising features observed upon dietary vitamin A deficiency (VAD), whereas those of *Rarb* or *Rarg* do not cause primary testis defects (Lufkin *et al*, 1993; Vernet *et al*, 2006).

Stem spermatogonia have remarkable ability to both self-renew and differentiate. The balance between these two processes is thought to depend on a proper environment, which is provided by the supporting SC (Payne and Braun, 2006; Ryu *et al*, 2006, and references therein). In addition, SC are essential to initiate spermatogenesis at puberty, and to maintain it after sexual maturity (Sharpe *et al*, 2003). SC display cyclical changes in morphology, gene expression and biochemical activity (Morales and Clermont, 1993; Parvinen, 1993), which are associated with stages of the seminiferous epithelium cycle, a series of constant germ cell associations reflecting coordination of meiosis and spermiogenesis (i.e., spermatid maturation; Russell *et al*, 1990). As the cyclical activity of SC is established before any signs of heterogeneity in germ cell populations, it may be involved in initiating spermatogenesis at puberty (Timmons *et al*, 2002). Given the central role of SC in spermatogonial stem cell self-renewal and spermatogenesis, we were interested in generating SC-specific *Rar* and *Rxr* knockouts. Using this genetic approach, we demonstrate that RARα is cell-autonomously instrumental to the cyclical activity of SC and to the structural integrity of the seminiferous epithelium, in contrast to RXRs, which are dispensable.

Results

SC-specific ablation of RARα in mice

Mice carrying *lox*P-flanked alleles of *Rara* (Chapellier *et al*, 2002a) were crossed with mice bearing the *Amh-Cre* transgene (Lecureuil *et al*, 2002) to generate *Rara*$^{Ser-/-}$ mutants, in which both alleles of *Rara* were excised in SC. These crosses also generated control males carrying two *lox*P-flanked alleles of *Rara*, which did not display histological defects and are hereafter referred to as wild-type (WT) mice. Importantly, no immunostaining for RARα was detected in the testes of *Rara*$^{Ser-/-}$ adult mice (Supplementary Figure 1), indicating that *Rara*$^{Ser-/-}$ mutants actually lack RARα in SC. Note that, as the *Amh–Cre* transgene is expressed from embryonic day 15.5 onwards (Lecureuil *et al*, 2002), excision of *Rara* occurs before the onset of spermatogenesis at postnatal day 5 (P5) (Bellve *et al*, 1977).

*Corresponding authors. NB Ghyselinck, IGBMC, 1 rue Laurent Fries, BP10142, Illkirch F-67404, France. Tel.: +33 388 655 674; Fax: +33 388 653 201; E-mail: norbert@igbmc.u-strasbg.fr and M Mark, IGBMC, 1 rue Laurent Fries, BP10142, Illkirch F-67404, France. Tel.: +33 388 655 636; Fax: +33 388 653 201; E-mail: marek@igbmc.u-strasbg.fr

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Ablation of RARα in SC results in reduced spermatogenesis and age-dependent testis degeneration

In young, 9-week-old $Rara^{Ser-/-}$ mutants ($n = 6$), spermatogenesis yielded mature spermatids in which nuclear elongation as well as acrosome and flagellum development was complete (St16; Figure 1F and Supplementary Figure 2). However, these mature spermatids failed to align at the luminal side of the seminiferous epithelium (St16; compare Figure 1E with F), and were often not released. They were instead retained within the epithelium (St16r; compare Figure 1G and H). In addition, they were scarce (St16;

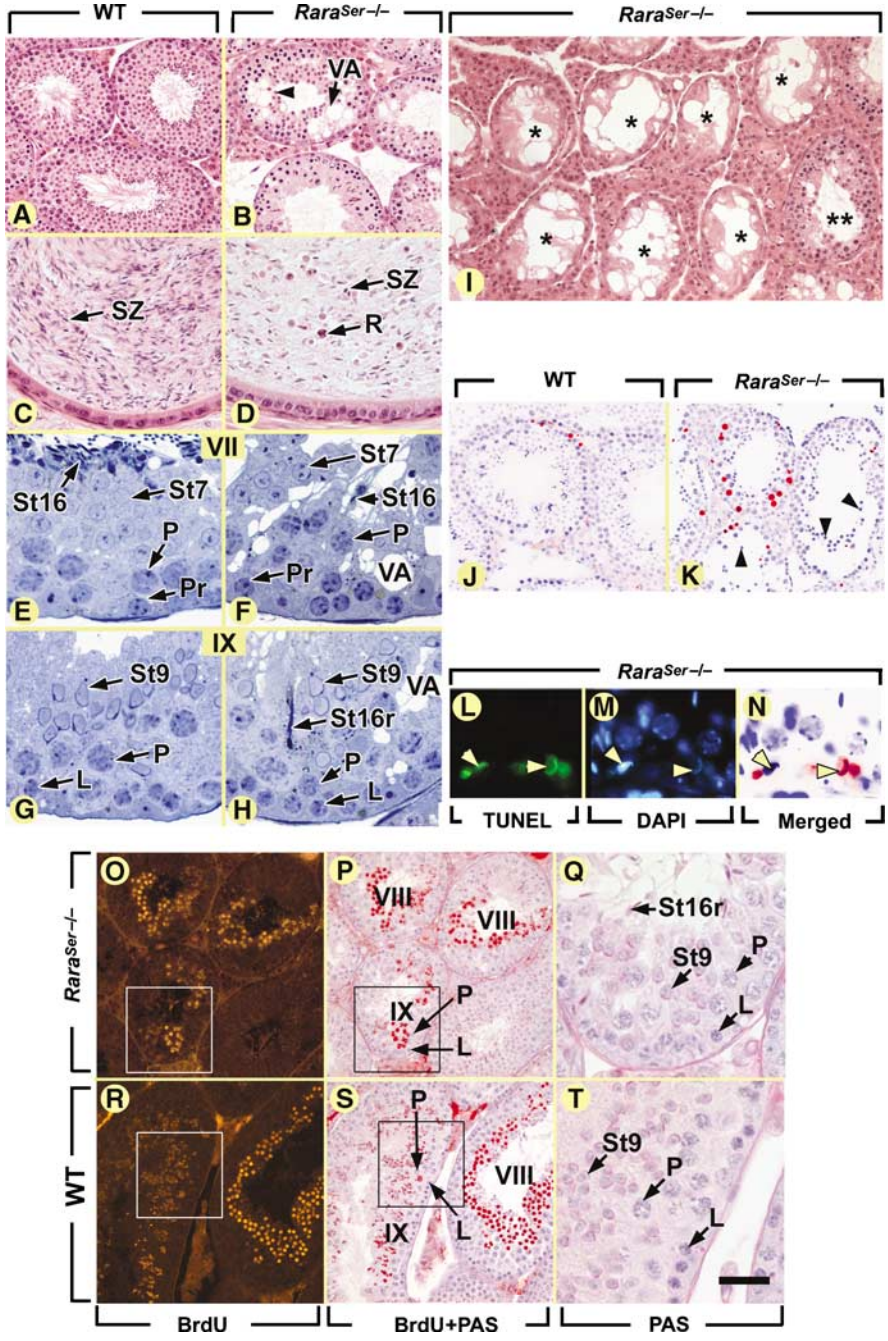

Figure 1 Ablation of RARα in SC yields a progressive testis degeneration resulting from spermatid desquamation and germ cell apoptosis, but not from a disruption of the seminiferous epithelium cycle. (**A–I**) Histological sections stained with hematoxylin and eosin (A–D, I) or toluidine blue (E–H) through the testes (A, B, E–I) or epididymides (C, D) of 9-week-old (A–H) and 12-month-old (I) mice. (**J–N**) TUNEL assays: note that in panels J, K and N, the positive signal was converted to a red false color and superimposed with the DAPI nuclear stain (blue false color). (**O–T**) Identification of the descendants of preleptotene spermatocytes, 18 days following a single injection of BrdU. (P) and (S) are superimpositions of the BrdU-labeled step 8 and 9 spermatids (red false color) with a periodic acid Schiff (PAS) counterstain; (Q) and (T) are high-magnification views of the boxed areas. L, leptotene spermatocytes; P, pachytene spermatocytes; Pr, preleptotene spermatocytes; R, round germ cells; St7, St9, St16, step 7, 9 and 16 spermatids, respectively; St16r, retained step 16 spermatids; SZ, spermatozoa; VA, vacuoles. Asterisks and double asterisks indicate tubules containing SC only and full complement of germ cells, respectively. The black and yellow arrowheads point to round spermatids detaching from the seminiferous epithelium and to TUNEL-positive elongated spermatids, respectively. Roman numerals designate stages of the seminiferous epithelium cycle. Bar (in T): 80 μm (A, B, I–K, O, P, R, S), 30 μm (C, D, Q, T), 20 μm (E–H) and 15 μm (L–N).

Table I Percentage of tubule cross-sections containing TUNEL-positive cells in 9-week-old WT and *Rara*$^{Ser-/-}$ mice, and distribution of apoptotic cell types

	WT	*Rara*$^{Ser-/-}$
Percentage of tubule sections containing TUNEL-positive round germ cells	18±1	58±12
Number of TUNEL-positive round germ cells in a testis cross-section	46±6	225±45
Percentage of spermatocytes	89±3	80±16
Percentage of spermatids	1±1	17±1
Percentage of unidentified	10±1	3±1

Mean±s.e.m.; $n=3$ in each group of age. The number of tubule sections analyzed in each testis was between 120 and 140.

compare Figure 1E and F), and frequently exhibited ultrastructural abnormalities indicative of necrosis (Supplementary Figure 2B). The seminiferous epithelium of *Rara*$^{Ser-/-}$ mutants also showed large vacuoles (VA; compare Figure 1A and B), and desquamation of round spermatids (black arrowhead in Figure 1B and K, compare with 1A and J). In keeping with these defects, the caudal epididymis contained low spermatozoa stores (compare SZ in Figure 1C and D), but numerous round spermatids (R; Figure 1D); all these cells were necrotic (Supplementary Figure 2J). Altogether, these data are indicative of complete but reduced spermatogenesis and testis degeneration (Holstein *et al*, 2003). A decreased production and a failure of detachment of mature spermatids contribute to the reduced spermatogenesis in *Rara*$^{Ser-/-}$ mutants. To investigate whether cell death also contributed to this phenotype, TUNEL assays were performed in 9-week-old testes (Table I). Numerous TUNEL-positive spermatocytes and round spermatids were detected in the mutant testes (compare Figure 1K with J), and TUNEL-positive elongated spermatids (yellow arrowheads, Figure 1L–N) were present in 68±1% (mean±s.e.m.; $n=3$ mutants) of the tubule sections versus 5±1% in WT testes ($n=3$). Thus, lack of RARα markedly impairs the SC capacity to support survival of meiotic (spermatocytes) and post-meiotic germ cells (spermatids).

In 12-month-old *Rara*$^{Ser-/-}$ mutants ($n=3$), up to 86% of tubule sections contained only SC (asterisks; Figure 1I and Supplementary Table I), indicating that (i) RARα in SC is also necessary for the survival of spermatogonia, and (ii) testis degeneration (i.e., vacuolation of the seminiferous epithelium, desquamation of immature spermatids and germ cell death) becomes more severe upon aging.

Ablation of RARα in SC does not affect their polarity and their density

At the ultrastructural level, SC of *Rara*$^{Ser-/-}$ mutants were essentially normal and the blood testis barrier appeared unaffected (Supplementary Figure 2). As a given SC can support survival and differentiation of only a limited number of germ cells (Sharpe *et al*, 2003), we assumed that germ cell desquamation and apoptosis observed in *Rara*$^{Ser-/-}$ mutants could be accounted for by a reduced SC density. However, no significant difference in SC density was noted between WT (26.7±0.8 cells/mm; mean±s.e.m.; $n=40$ tubule sections) and *Rara*$^{Ser-/-}$ testes (28.7±1.1 cells/mm; $n=44$ tubule sections) at 9 weeks of age (Supplementary Figure 4), indicating that the ratio of SC to germ cells is normal in *Rara*$^{Ser-/-}$ mutants.

Ablation of RARα in SC does not affect the cycle of the seminiferous epithelium or the duration of spermatogenesis

The different generations of germ cells, while synchronously progressing through spermatogenesis, form cellular associations of fixed composition (called epithelial stages) that follow each other according to a stereotyped sequence known as the seminiferous epithelium cycle. Twelve epithelial stages (I–XII) can be identified in the mouse (Russell *et al*, 1990). In 9-week-old *Rara*$^{Ser-/-}$ testes, the 12 epithelial stages were readily identifiable and each occupied the circumference of a seminiferous tubule cross-section (Figure 1E–H). The duration of meiotic and post-meiotic phases of spermatogenesis was evaluated by identifying, 18 days after a single injection of BrdU, the labeled descendants of preleptotene spermatocytes. In both WT and *Rara*$^{Ser-/-}$ mice, the most advanced BrdU-labeled cells were step 9 spermatids, and there was no labeling in germ cells younger than step 8 spermatids (Figure 1O–T). These data indicate that the absence of RARα in SC does not alter the seminiferous epithelium cycle or the duration of meiosis and spermiogenesis.

Absence of RARα or of RA in SC delays the progression of the prepubertal wave of spermatogenesis

To analyze the impact of the *Rara*$^{Ser-/-}$ mutation on the prepubertal wave of spermatogenesis, which is normally completed by postnatal day 35 (P35), we compared development of the seminiferous epithelium in *Rara*$^{Ser-/-}$ and WT males ($n=3$ for each genotype and age group) at P5 (i.e., when gonocytes differentiate into primitive A spermatogonia), P10 (i.e., at the onset of meiosis), P20 (i.e., when post-meiotic cells first appear), P25 and P30 (Bellve *et al*, 1977).

At P5, *Rara*$^{Ser-/-}$ and WT seminiferous cords were morphologically indistinguishable (not shown), although the cords expressing the spermatogonia differentiation marker *Stra8* were significantly fewer in *Rara*$^{Ser-/-}$ testes (Figure 2A and Supplementary Figure 5). At P10, leptotene spermatocytes, which represented the most advanced germ cell type, were also fewer in *Rara*$^{Ser-/-}$ testes (Figure 2B). At P20, a majority of *Rara*$^{Ser-/-}$ tubule sections did not contain spermatocytes beyond the zygotene stage, whereas the vast majority of their WT counterparts displayed more advanced pachytene and diplotene spermatocytes (Figure 2C). Along the same lines, post-meiotic round spermatids were absent in *Rara*$^{Ser-/-}$ testes at P20, but were always present in age-matched WT testes (Figure 2C). These data are indicative of a delay in spermatogenesis, which interestingly is not related to testis degeneration as increase in germ cell apoptosis (Figure 2D) and vacuolation of the seminiferous epithelium (not shown) were not observed in *Rara*$^{Ser-/-}$ testes before P20 and P25, respectively.

Androgen and FSH signaling pathways in SC play essential functions in testis development, as inactivation of androgen and FSH receptors (*Ar* and *Fshr*, respectively) delay the prepubertal wave of spermatogenesis (Chang *et al*, 2004; De Gendt *et al*, 2004; Johnston *et al*, 2004). However, these two pathways are not involved in the delay of prepubertal spermatogenesis in *Rara*$^{Ser-/-}$ mutants as the expression pattern of *Ar* (Figure 2G–J) and *Fshr* (Supplementary Figure 5) were normal in immature SC of *Rara*$^{Ser-/-}$ testes.

To investigate whether the delay in spermatogonia differentiation observed in testes lacking RARα could be mimicked

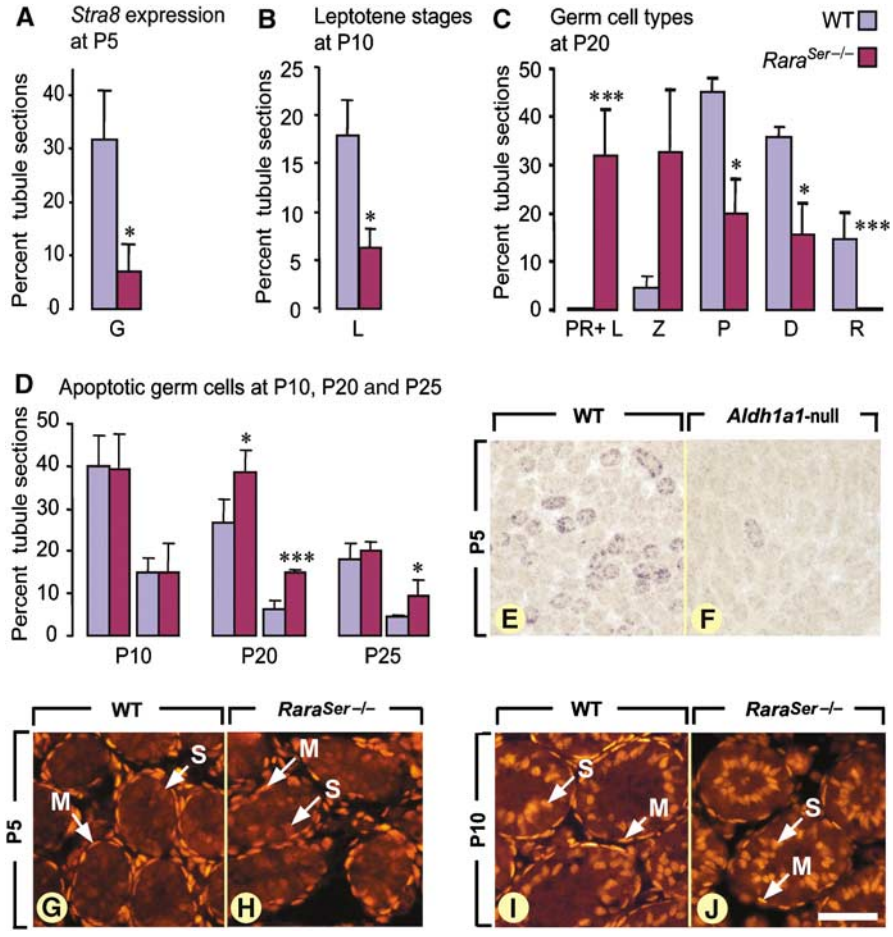

Figure 2 Ablation of RA signaling in SC delays the first spermatogenic cycle without altering the normal timing of androgen receptor expression. (**A**) Percentage of seminiferous cord cross-sections containing *Stra8*-positive spermatogonia in WT (blue bars) and in $Rara^{Ser-/-}$ (purple bars) testes at P5. (**B, C**) Percentages of seminiferous cord or tubule cross-sections in which leptotene spermatocytes (L), preleptotene/leptotene (PR + L), zygotene (Z), pachytene (P) and diplotene (D) spermatocytes and round spermatids (R) represent the most advanced germ cell types in WT (blue bars) and $Rara^{Ser-/-}$ (purple bars) testes at P10 (B) and P20 (C). (**D**) Percentages of seminiferous cord or tubule cross-sections containing at least one (first set of bars) or at least three (second set of bars) apoptotic germ cell in WT (blue bars) and in $Rara^{Ser-/-}$ (purple bars) testes at P10, P20 and P25. Note that in panels A–D, the bars represent mean ± s.e.m. ($n = 3$–5); the asterisks indicate a significant difference ($^{*}P < 0.05$; $^{***}P < 0.001$). (**E, F**) Detection of *Stra8* transcripts at P5 in WT and *Aldh1a1*-null testes: although histologically indistinguishable at this developmental stage, the seminiferous cord sections containing *Stra8*-positive spermatogonia are much less abundant in the *Aldh1a1*-null than in the WT testis. (**G–J**) Immunodetection of androgen receptor (red signal) at the onset of spermatogenesis (i.e., P5) and at the beginning of meiosis (i.e., P10). At P5, the androgen receptor is detected in all peritubular myoid cell precursors, as well as occasionally and weakly in immature SC. At P10, the androgen receptor is expressed in peritubular myoid cells and in all immature SC. G, spermatogonia; PR, L, Z, P, D, preleptotene, leptotene, zygotene, pachytene and diplotene spermatocytes, respectively; M, peritubular myoid cells. S, immature Sertoli cells; R, spermatids. Bar (in J): 200 μm (E, F) and 50 μm (G–J).

upon decreasing RA availability, we analyzed expression of *Stra8* in the testes of *Aldh1a1*-null mice lacking retinaldehyde dehydrogenase 1 (Matt *et al*, 2005), which is the main RA-synthesizing enzyme in SC at P5 (Vernet *et al*, 2006). In *Aldh1a1*-null testes at P5, only 4 ± 3% (mean ± s.e.m.; $n = 3$) of seminiferous cords contained spermatogonia expressing *Stra8* versus 28 ± 3% (mean ± s.e.m.; $n = 3$) in WT littermates (compare Figure 2E with F). Therefore, a RA-liganded RARα in SC is required for proper spermatogonia differentiation during the prepubertal wave of spermatogenesis.

Cyclical expression of numerous genes is lost in SC lacking RARα

The cyclical activity of SC was investigated through analysis of genes known to display SC-restricted cyclical expression, such as the androgen receptor (AR) whose expression peaks at stage VI–VIII (Figure 3A–D; Zhou *et al*, 2002), and the membrane protein STRA6 expressed at stages VII–VIII and IX (Figure 4A and B; Bouillet *et al*, 1997). In $Rara^{Ser-/-}$ adults, all SC displayed similar levels of AR (Figure 3E–H), and low, uniform, levels of STRA6 from stage I to stage XII (Figure 4C and D). Along these lines, the stage-dependent variations of expression of GATA1 (Yomogida *et al*, 1994), galectin-1 (Timmons *et al*, 2002), clusterin (Morales *et al*, 1987) and procathepsin L (Wright *et al*, 2003) were all lost in the $Rara^{Ser-/-}$ seminiferous epithelium (Supplementary Figures 6 and 7). Altogether, these data indicate that the cyclical activity of SC is abolished in $Rara^{Ser-/-}$ mutants. Importantly, the cyclical expression of *Stra8* in spermatocytes and A spermatogonia (Figure 4E–H; Supplementary Figure 7) and *Rxra* in round spermatids (not shown) were not modified in $Rara^{Ser-/-}$ testes. These observations are in keeping with our histological findings that ablation of RARα in SC does not alter the seminiferous epithelium cycle (Figure 1E–H).

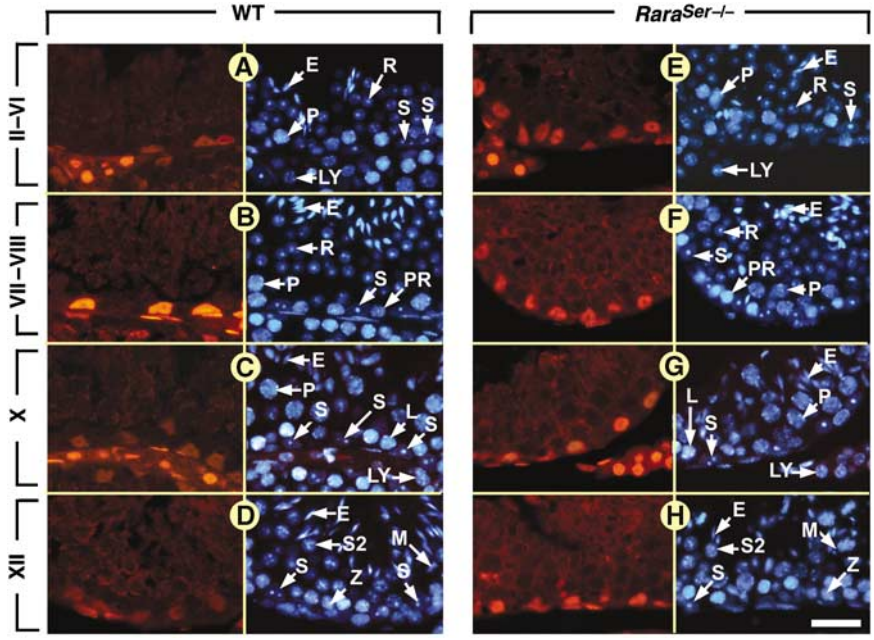

Figure 3 Ablation of RARα in SC abrogates the epithelial stage-dependent variations of AR expression. Immunohistochemical detection of AR in the seminiferous epithelium at 9 weeks of age. (**A–D**) In WT testis, immunolabeling for AR is strong in SC nuclei at stages VII and VIII and weak at other epithelial stages. (**E–H**) In *Rara*$^{Ser-/-}$ mutant testis, AR is expressed at similar levels in all SC nuclei, irrespective of the epithelial stage. Note that (i) the left side of each panel corresponds toe staining using the anti-AR antibody and the right side to a DAPI nuclear counterstain and (ii) the histological sections from WT males and from *Rara*$^{Ser-/-}$ mutants were processed in parallel for immunohistochemistry, and identical exposure times were used to acquire the fluorescence pictures. E, elongated spermatids; L, leptotene spermatocytes; LY, Leydig cell; M, spermatocytes in metaphase; P, pachytene spermatocytes; PR, preleptotene spermatocytes; R, round spermatids; S, Sertoli cells; S2, type 2 spermatocytes; Z, zygotene spermatocytes. Roman numerals designate stages of the seminiferous epithelium cycle: II–VI, stage II, III, IV, V or VI; VII–VIII, stage VII or VIII. Bar: 30 μm (A–H).

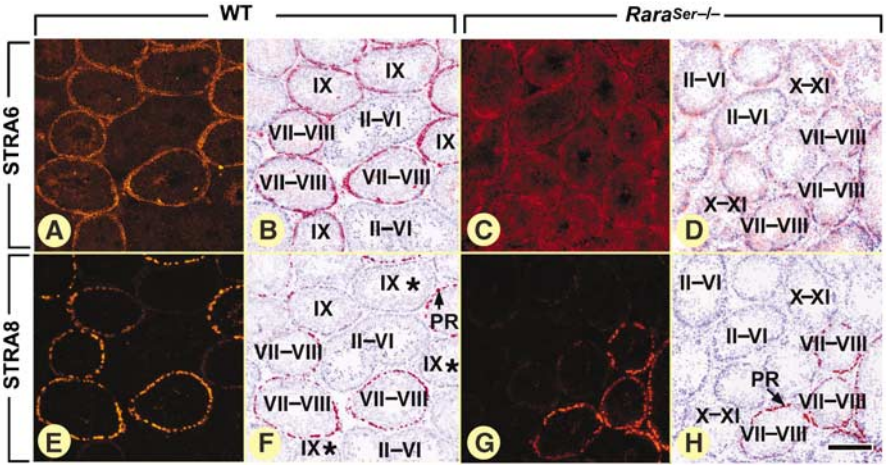

Figure 4 Ablation of RARα in SC abrogates the epithelial stage-dependent variations of *Stra6* expression, but does not alter cyclic expression of the germ cell marker *Stra8* in adult testes. Immunostaining for STRA6 (**A–D**) and STRA8 (**E–H**) in WT and *Rara*$^{Ser-/-}$ testes, as indicated. In WT males, STRA6 protein is present at epithelial stages VII–IX, and its level peaks at stage VIII. In WT testes, immunolabeling for STRA8 is strong in preleptotene spermatocytes (present at stages VII–VIII of the seminiferous epithelium cycle) and weak in leptotene spermatocytes (present at stages IX–X), and STRA8-containing spermatocytes co-distribute with STRA6-containing SC. Note that STRA8 is also expressed in spermatogonia (see Supplementary Figure 7). In *Rara*$^{Ser-/-}$ testes, the epithelial stage-specific expression of STRA6 is lost; in contrast, STRA8 distribution is unaffected. Note that (i) panels A, B and panels E, F correspond to consecutive sections of a WT testis and (ii) that panels C, D and panels G, H correspond to consecutive sections of a *Rara*$^{Ser-/-}$ testis: PR, preleptotene spermatocytes. Roman numerals designate stages of the seminiferous epithelium cycle: II–VI, stage II, III, IV, V or VI; VII–VIII, stage VII or VIII; X–XI, stage X or XI. Asterisks indicate tubule sections containing STRA8-positive leptotene spermatocytes, which are not visible at the illustrated magnification. In panels B, D, F and H, the immunohistochemical signals were converted to a red false color and superimposed with the DAPI nuclear counterstain (blue false color). Bar (in H): 160 μm (A–H).

We also analyzed the cyclical activity of SC before the appearance of the seminiferous epithelium cycle. In WT males at P5 and P10, distribution of STRA6 protein, and the cellular retinol-binding protein CRBP1 (*Rbp1*) and galectin-1 (*Lgals1*) transcripts varied between seminiferous cord sections (Figure 5A–C and G–I). In contrast, expression of

these genes appeared uniform in all seminiferous cords of *Rara*$^{Ser-/-}$ mutants (Figure 5D–F and J–L). Importantly, the total amounts of *Stra6*, *Lgals1* and *Rbp1* transcripts measured by quantitative RT–PCR in *Rara*$^{Ser-/-}$ testes at P5 did not significantly differ from those in WT testes (Supplementary Figure 5), indicating that changes in their expression were only qualitative. The GATA1 immunolabeling at P10 varied markedly between individual seminiferous cords in WT testes, whereas it was strong in all SC nuclei in *Rara*$^{Ser-/-}$ testes (compare Figure 5M and N). This abnormal, uniform, expression of GATA1 was also observed in *Rara*$^{Ser-/-}$ testes at P20 (compare Figure 5O and P), indicating that it was not related to the developmental delay of the mutant testis (see above). Altogether, these data demonstrate that the cyclical activity of SC is disrupted in *Rara*$^{Ser-/-}$ mutants, already at the onset of pubertal testis development.

Similar to the adult situation (see above), loss of *Stra6* cyclical expression in prepubertal *Rara*$^{Ser-/-}$ testes (Figure 5D and J) did not abolish the cyclical expression of *Stra8* (not shown). This finding was quite unexpected, as in prepubertal WT testes we found a strong positive, temporal and spatial, correlation between SC displaying high levels of STRA6- and STRA8-positive spermatogonia and spermatocytes (Supplementary Figure 8). The coordinated expression of *Stra6* and *Stra8* is therefore uncoupled upon ablation of *Rara* in SC.

Additional ablation of RARβ and RARγ in SC does not increase the severity of the phenotype resulting from RARα ablation

A striking variability in the extent of the seminiferous epithelium vacuolation was observed not only in different *Rara*$^{Ser-/-}$ mutants but also within a given mutant in different tubule

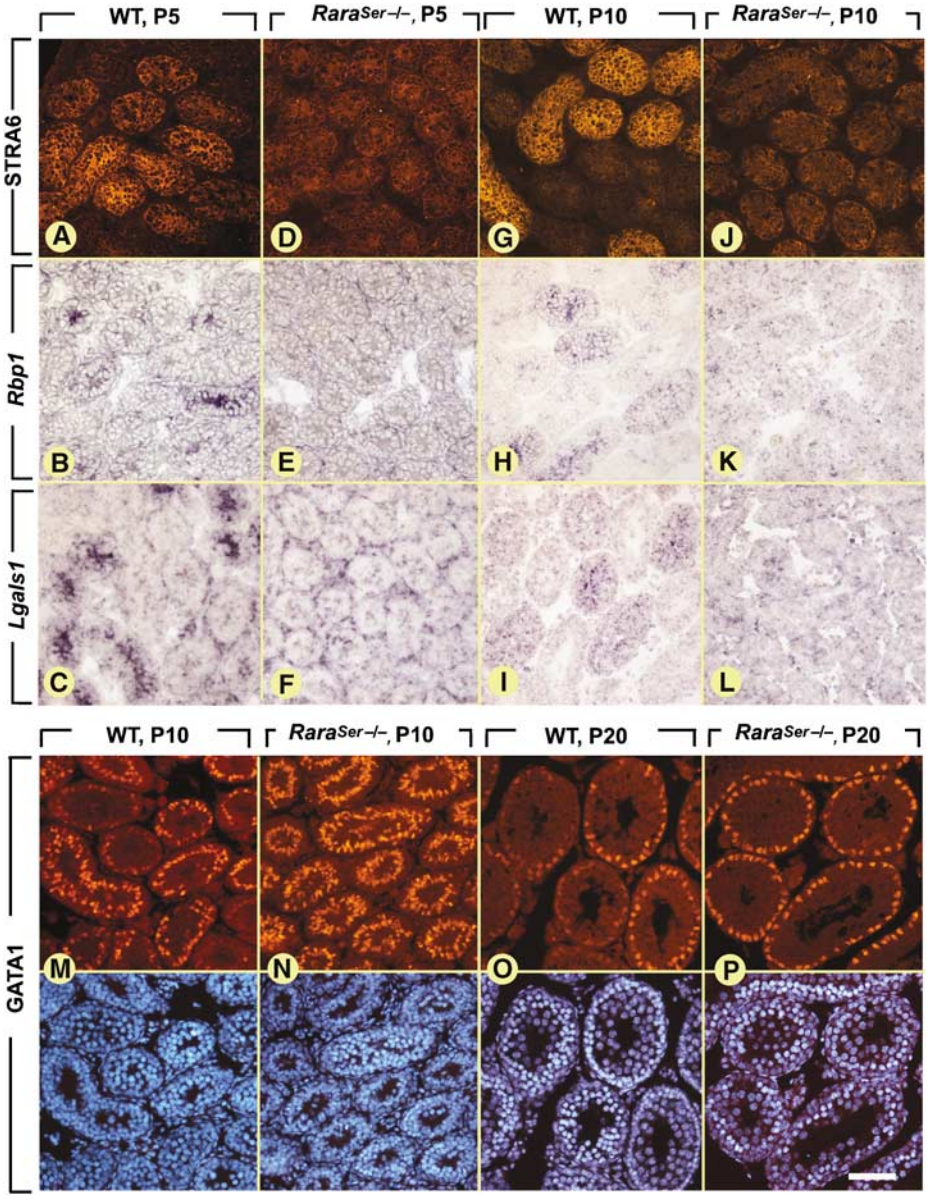

Figure 5 Ablation of RARα in SC abrogates the epithelial stage-dependent variations of gene expression in prepubertal testes. (**A, D, G, J**) Immunostaining for STRA6. (**B, C, E, F, H, I, K, L**) ISH analyses using *Rbp1* (B, E, H, K) and *Lgals1* (C, F, I, L) antisense probes; the positive signals for transcripts are violet. (**M–P**) Immunostaining for GATA1. (A, D, G, J, M–P) The positive signal for STRA6 and GATA1 is red. The DAPI counterstain is also illustrated in panels M–P. Bar (in P): 50 μm (A–F) and 80 μm (G–P).

segments (Figure 1I and Supplementary Figure 3). Although RARα is the only RAR evidenced in SC using immunohistochemistry (Vernet *et al*, 2006), the variability in seminiferous epithelium degeneration left open the possibility that stochastic variations of RARβ and/or RARγ possibly present in low (i.e., undetectable) amounts could compensate for the RARα loss of function. To investigate this possibility, we generated *Rara/b/g*$^{Ser-/-}$ mice lacking RARα, RARβ and RARγ in SC (see Supplementary information). Testes from 9-week-old *Rara/b/g*$^{Ser-/-}$ mutants ($n=3$) displayed alterations that were indistinguishable from those found in *Rara*$^{Ser-/-}$ mice (Supplementary Figure 9), indicating that RARα is the sole functional RAR in SC.

Ablation of all RXRs in SC does not recapitulate the Rara$^{Ser-/-}$ *phenotype*

RXRβ is the predominant RXR in SC (Vernet *et al*, 2006). Selective ablation of *Rxrb* in SC (*Rxrb*$^{Ser-/-}$ mutation) yielded, at 9 weeks of age, testis defects identical to those generated upon inactivation of RXRβ function in the whole organism (Kastner *et al*, 1996; Mascrez *et al*, 2004), namely an accumulation of lipids in SC and a failure of spermiation (our unpublished data). The latter is also generated upon ablation of *Rara* (see above). On the other hand, testis degeneration (i.e., vacuolation, desquamation of immature round spermatids, increased germ cell apoptosis) and loss of *Stra6* cyclical expression, which are hallmarks of age-matched *Rara*$^{Ser-/-}$ mutants, were never observed in *Rxrb*$^{Ser-/-}$ mutants (not shown). To exclude the possibility that RXRα and/or RXRγ present at low amounts in SC could compensate for the loss of RXRβ, mice lacking RXRα, RXRβ and RXRγ in SC (i.e., *Rxra/b*$^{Ser-/-}$*/Rxrg*-null mutants, see Supplementary information) were analyzed. At 9 weeks of age, these mutants ($n=3$) recapitulated the defects of *Rxrb*$^{Ser-/-}$ mutants (Figure 6D), but did not exhibit degeneration of the seminiferous epithelium (Figure 6B and F) and loss of *Stra6* cyclical expression (Figure 6H; not shown). Interestingly, none of the mutations altered the cyclical expression of *Stra8* in germ cells (Figure 6I and J). Therefore, in contrast to RARα, RXRs are dispensable for the structural integrity of the seminiferous epithelium and for the cyclical activity of SC.

Discussion

All functions of RARα in the mouse testis are cell-autonomously exerted in SC

RARα expression studies had left open the possibility that it could have exerted functions on testis physiology by acting in germ cells (Akmal *et al*, 1997; Gaemers *et al*, 1997) or in cells of the hypothalamus–pituitary axis (Krezel *et al*, 1999). However, the present study demonstrates that the failure of

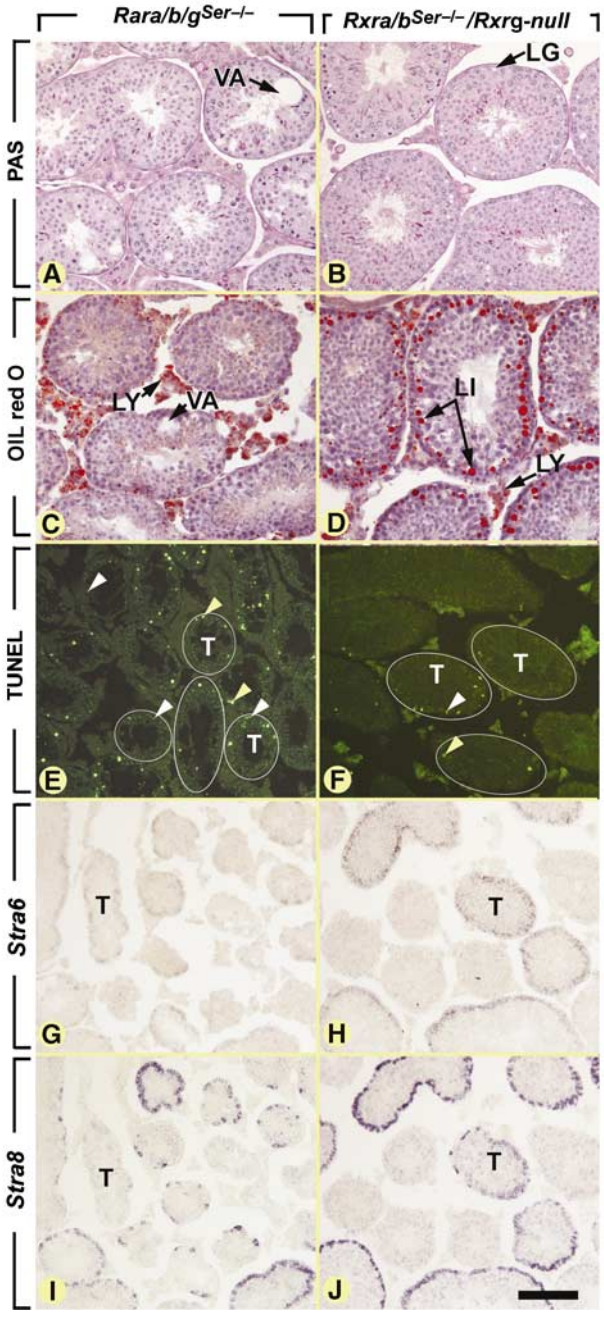

Figure 6 Ablations of all three RARs (i.e., *Rara/b/g*$^{Ser-/-}$ mutants) or of all three RXRs (i.e., *Rxra/b*$^{Ser-/-}$*/Rxrg*-null mutants) yield very different abnormalities. Histological sections of testes at 9 weeks of age stained by (**A, B**) the PAS method, (**C, D**) oil red O for detection of lipids droplets (red dots), (**E, F**) the TUNEL method to detect apoptopic cells (green fluorescent signals) and (**G–J**) ISH using antisense probes to (G, H) *Stra6* and (I, J) *Stra8* (purple signals). (A, C, E, G, I) The *Rara/b/g*$^{Ser-/-}$ mutant testis displays (i) large, lipid-free, vacuoles (VA in panel A), (ii) numerous apoptotic round germ cells (some indicated by yellow arrowheads in panel E) and elongated spermatids (indicated by white arrowheads in panel E) in numerous seminiferous tubules sections (T) and (iii) low and uniform expression of *Stra6* in all seminiferous tubules that does not follow the cyclical expression of *Stra8* shown on an consecutive section. (B, D, F, H, J) In contrast, the *Rxra/b*$^{Ser-/-}$*/Rxrg*-null mutant testis displays (i) numerous lipid inclusions (LI in panel D) whose extraction during paraffin embedding yields 'lipid ghosts' (LG in panel B) (ii) a normal, low, proportion of apoptotic round germ cells (yellow arrowheads in panel F), but an increase in the proportion of TUNEL-positive elongated spermatids (white arrowheads; see Mascrez *et al*, 2004 for further details), when compared to WT mice and (iii) normal cyclic expression of *Stra6* in seminiferous tubules superimposed with that of *Stra8* shown on an adjacent section. (E, F) Some tubule sections were highlighted by a thin white line. LG, lipid ghost; LI, lipid inclusion; LY, Leydig cell; T, seminiferous tubule; VA, vacuoles. Bar (in J): 80 μm (A–D) and 160 μm (E–J).

spermiation, epithelial vacuolation, germ cell desquamation and apoptosis displayed by *Rara*$^{Ser-/-}$ and by *Rara*-null mutants are indistinguishable (Lufkin *et al*, 1993). In addition, the onset of degeneration at a prepubertal stage following cessation of SC proliferation (Vergouwen *et al*, 1991), its progression with aging to ultimately yield seminiferous tubules with only SC and the delay in the first spermatogenic cycle are identical in *Rara*$^{Ser-/-}$ and *Rara*-null mutant mice. These observations therefore demonstrate that all functions of RARα in testis development and spermatogenesis are cell-autonomously exerted in SC.

An RARα-mediated RA signaling pathway in prepubertal but not adult SC promotes spermatogonia differentiation

Rara$^{Ser-/-}$ mutants exhibit a delay in the progression of the first spermatogenetic wave. This delay is established during spermatogonia differentiation, as indicated by the retarded expression of *Stra8*. It is unlikely to additionally affect meiosis and spermiogenesis, as the time required to generate elongated spermatids from preleptotene spermatocytes is normal in the sexually mature *Rara*$^{Ser-/-}$ testis. Moreover, it cannot be accounted for by the increase in germ cell apoptosis, which occurs later during development of the *Rara*$^{Ser-/-}$ testis. On the other hand, RA binding to RARα is most likely required, as a developmental delay in the appearance of *Stra8*-positive spermatogonia is observed in *Aldh1a1*-null mice. Thus, a cell-autonomous effect of RA-liganded RARα in immature SC is required to promote spermatogonia differentiation during the prepubertal spermatogenic wave.

As mentioned above, RARα promotes differentiation of spermatogonia during prepuberty. In contrast, spermatogonia differentiation arrest during adulthood, which is the hallmark of the VAD-induced testis degeneration, is not observed upon RAR ablation in SC. For instance, retention of BrdU in spermatogonia and disappearance of entire germ cell layers in the seminiferous tubule sections (Ghyselinck *et al*, 2006) are absent in *Rara*$^{Ser-/-}$ and *Rara/b/g*$^{Ser-/-}$ mutants (present report). In keeping with these observations, expression of the RA target gene *Stra8*, which is required for meiotic initiation, is maintained in *Rara*$^{Ser-/-}$ and *Rara/b/g*$^{Ser-/-}$ testes, whereas it is abolished in spermatogonia from VAD testes (Bowles *et al*, 2006; Ghyselinck *et al*, 2006; Koubova *et al*, 2006 and present report). Therefore, inhibition of RAR signaling solely in SC cannot account for the VAD-induced arrest in spermatogonia differentiation.

RARα signaling is a master regulator required to initiate the cyclical activity of SC, which is however not instrumental to the seminiferous epithelium cycle

That expression of *Lgals1, Rbp1, Stra6* and *Gata1* is no longer cyclical in postnatal mitotic SC lacking RARα indicates that this nuclear receptor may initiate the cyclical activity of these cells. In addition, adult post-mitotic SC lacking RARα have also lost their capacity to modulate expression of *Ar, Ctsl* and *Clu*, strongly suggesting thereby that RARα acts on top of a genetic cascade orchestrating the cyclical activity of SC.

It has been proposed that the progression of germ cells through the stages of the seminiferous epithelium cycle modulates expression of specific genes in SC, which, in turn, may ultimately influence the development of the surrounding germ cells (Yomogida *et al*, 1994; Griswold, 1995; Bitgood *et al*, 1996; Zhao *et al*, 1996; Zabludoff *et al*, 2001). As a matter of fact, we show that the 12 stages of the seminiferous epithelium cycle are easily identified in *Rara*$^{Ser-/-}$, *Rara/b/g*$^{Ser-/-}$ and *Rara*-null mutants and, as for WT testes, each stage occupies the whole circumference of a tubule section. In addition, the normal, epithelial stage-specific expression of the germ cell markers *Stra8* and *Rxra* is maintained in *Rara*$^{Ser-/-}$ mutants, and BrdU incorporated into premeiotic spermatocytes of *Rara*$^{Ser-/-}$ mutants is transferred to homogeneous cohorts of spermatids at the same step of maturation as in WT mice. These observations do not support the view that RAR signaling in SC is critical for the maintenance of proper germ cell associations (Chung *et al*, 2004), and clearly indicate that, unexpectedly, cyclical gene expression by SC is not essential to coordinate the cyclical progression of germ cells through meiosis and spermiogenesis. We additionally show that co-distribution of SC expressing *Stra6* and germ cells expressing *Stra8* is set up along the seminiferous cords already at P5 (see Supplementary Figure 8). This indicates that coordination between germ cell differentiation and cyclical activity of SC precedes the onset of spermatogonia differentiation, and therefore occurs much earlier than previously thought (Timmons *et al*, 2002).

In any events ablation of RAR signaling in SC causes germ cell apoptosis and desquamation. These seminiferous epithelium dysfunctions may be accounted for by the disruption of SC cyclical gene expression, which precedes testis degeneration by about 2 weeks. In this context, ablations of *Clu, Ctsl* and disruption of androgen signaling cause defects of spermatogenesis, which, when taken altogether, are reminiscent of those displayed by *Rara*$^{Ser-/-}$ mutants (Bailey *et al*, 2002; Wright *et al*, 2003; Chang *et al*, 2004; De Gendt *et al*, 2004). On the other hand, despite the fact that targeted inactivation of *Gata1, Lgals1* and *Rbp1* does not yield reproductive phenotypes (Poirier and Robertson, 1993; Ghyselinck *et al*, 1999; Lindeboom *et al*, 2003), the possibility exists that their simultaneous ablation may impair SC functions.

RARα in SC exerts physiological functions, even in the absence of RXRs

The convergence of phenotypes generated upon *Rar* and *Rxr* ablations has clearly shown that RXR/RAR heterodimers are the functional units transducing the RA signal in the mouse, both during development and in adult tissues (Chapellier *et al*, 2002b; Calleja *et al*, 2006; Mark *et al*, 2006). In the present study, we definitely demonstrate that there is no functional redundancy among RAR isotypes and among RXR isotypes in SC as it is the case in embryonic tissues (reviewed in Mark *et al*, 2006), and therefore that RARα and RXRβ are the sole functional RA receptors in SC. Interestingly, except for spermiation defects, *Rara*$^{Ser-/-}$ and *Rxrb*$^{Ser-/-}$ testes exhibit distinct sets of abnormalities. Those observed in *Rxrb*$^{Ser-/-}$ mutants can be ascribed to a loss of RXRβ/LXRβ-mediated events (Mascrez *et al*, 2004). On the other hand, the testis degeneration observed in *Rara*$^{Ser-/-}$ but not in *Rxrb*$^{Ser-/-}$ mutants reveals for the first time that RARα can exert functions *in vivo*, independently of RXRs. This conclusion could have not been reached without having inactivated all three *Rxr* genes in SC. This was actually the only experimental way to definitely rule out a functional compensation of RXRβ functions by RXRα and/or RXRγ, which might have been expressed at low, undetectable levels in these cells. This

finding opens up new perspectives on physiological functions of RARs, which, as suggested from *in vitro* studies, may involve regulation of gene expression through homodimers, heterodimers with vitamin D3 and thyroid hormone receptors, or repression of the AP-1 transcription complexes activity (Chambon, 1996; Garcia-Villalba *et al*, 1996; Benkoussa *et al*, 2002; Conde *et al*, 2004; Lee and Privalsky, 2005 and references therein).

Understanding spermatogenesis is the prerequisite to develop concepts for reprogramming spermatogonia to therapeutic stem cells (Guan *et al*, 2006). RA plays crucial roles in the differentiation of stem germ cells (Bowles *et al*, 2006; Koubova *et al*, 2006). In this context, our results provide the first evidence that this developmental hormone signal controls in-SC processes that are required for spermatogenesis. They also demonstrate that RARα on its own, that is, in the absence of an RXR heterodimeric partner, is a master transcriptional regulator of the cyclical activity of SC.

Materials and methods

Histology

Histological observations, as well as analyses involving immunohistochemistry (IHC) and *in situ* hybridization (ISH), were repeated on at least three mice per age group. Testes and epididymides destined for paraffin embedding were fixed in Bouin's fluid for 48 h; sections were stained with either hematoxylin and eosin or the periodic acid Schiff (PAS) method. Testes and epididymides destined for epon embedding were perfusion-fixed with 2.5% glutaraldehyde (w/v) in PBS and processed as described (Kastner *et al*, 1996); semi-thin (i.e., 1 μm thick) sections were stained with toluidine blue. Detection of BrdU incorporation and apoptotic cells were as described (Ghyselinck *et al*, 2006; Vernet *et al*, 2006).

Immunohistochemistry and in situ hybridization analyses

For immunodetection of STRA6, 10-μm-thick sections of freshly frozen testes were post-fixed for 5 min in cold acetone at −20°C, air-dried, hydrated in PBS and fixed for a second time in ice-cold 4% (w/v) paraformaldehyde in PBS. For immunodetection of STRA8, the fixation step in acetone was omitted. For immunodetection of AR and GATA1, testes were fixed by intracardiac perfusion of ice-cold 4% (w/v) paraformaldehyde in PBS, then kept in the same fixative overnight at 4°C, washed in PBS, dehydrated and embedded in paraffin. The sections were incubated overnight at 4°C with the anti-STRA6 antibody (Bouillet *et al*, 1997) diluted 1:100 in PBS, anti-STRA8 antibody (Oulad-Abdelghani *et al*, 1996) diluted 1:500 anti-AR rabbit polyclonal antibody (sc-816; Santa Cruz Biotechnologies) diluted 1:500, or anti-GATA1 rat monoclonal antibody (sc-265; Santa Cruz Biotechnologies) diluted 1:100. Detection of bound primary antibodies was achieved by incubating the section for 45 min at room temperature using either a Cy3-conjugated goat anti-rabbit IgG (Biomol Immuno Research Laboratories, Exeter, UK) diluted 1:500, or a Cy3-conjugated goat anti-rat IgG (Jackson Immunoresearch Laboratories, Baltimore, PA) diluted 1:200. As a control of specificity, the anti-AR antibody was incubated with a histological section from an *Ar*-null mutant (not shown).

ISH with digoxigenin-labeled probes was as described (Vernet *et al*, 2006). The plasmids containing full-length *Rbp1* (650 bp long) and *Stra8* (1180 bp long) cDNAs, or parts of *Stra6* (244 bp long; exons 5–7), *Lgals1* (366 bp long; exons 2–4), *Clu* (942 bp long; exons 5–9) and *Ctsl* (925 bp long; exons 2–8) cDNAs were linearized and used as templates for the synthesis of sense or antisense riboprobes.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Akmal KM, Dufour JM, Kim KH (1997) Retinoic acid receptor alpha gene expression in the rat testis: potential role during the prophase of meiosis and in the transition from round to elongating spermatids. *Biol Reprod* **56:** 549–556

Bailey RW, Aronow B, Harmony JA, Griswold MD (2002) Heat shock-initiated apoptosis is accelerated and removal of damaged cells is delayed in the testis of clusterin/ApoJ knock-out mice. *Biol Reprod* **66:** 1042–1053

Benkoussa M, Brand C, Delmotte MH, Formstecher P, Lefebvre P (2002) Retinoic acid receptors inhibit AP1 activation by regulating extracellular signal-regulated kinase and CBP recruitment to an AP1-responsive promoter. *Mol Cell Biol* **22:** 4522–4534

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M (1977) Spermatogenic cells of the prepuberal mouse isolation and morphological characterization. *J Cell Biol* **74:** 68–85

Bitgood MJ, Shen L, McMahon AP (1996) Sertoli cell signaling by desert hedgehog regulates the male germline. *Curr Biol* **6:** 298–304

Bouillet P, Sapin V, Chazaud C, Messaddeq N, Decimo D, Dolle P, Chambon P (1997) Developmental expression pattern of Stra6 a retinoic acid-responsive gene encoding a new type of membrane protein. *Mech Dev* **63:** 173–186

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. *Science* **312:** 596–600

Calleja C, Messaddeq N, Chapellier B, Yang H, Krezel W, Li M, Metzger D, Mascrez B, Ohta K, Kagechika H, Endo Y, Mark M, Ghyselinck NB, Chambon P (2006) Genetic and pharmacological evidence that a retinoic acid cannot be the RXR-activating ligand in mouse epidermis keratinocytes. *Genes Dev* **20:** 1525–1538

Chambon P (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J* **10:** 940–954

Chambon P (2005) The nuclear receptor superfamily: a personal retrospect on the first two decades. *Mol Endocrinol* **19:** 1418–1428

Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H, Yeh S (2004) Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in sertoli cells. *Proc Natl Acad Sci USA* **101:** 6876–6881

Chapellier B, Mark M, Garnier JM, LeMeur M, Chambon P, Ghyselinck NB (2002a) A conditional floxed (loxP-flanked) allele for the retinoic acid receptor alpha (RARalpha) gene. *Genesis* **32:** 87–90

Chapellier B, Mark M, Messaddeq N, Calleja C, Warot X, Brocard J, Gérard C, Li M, Metzger D, Ghyselinck NB, Chambon P (2002b) Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled. *EMBO J* **21:** 3402–3413

Chung SS, Sung W, Wang X, Wolgemuth DJ (2004) Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. *Dev Dyn* **230:** 754–766

Conde I, Paniagua R, Fraile B, Ruiz A, Arenas MI (2004) Expression of vitamin D3 receptor and retinoid receptors in human breast cancer: identification of potential heterodimeric receptors. *Int J Oncol* **25:** 1183–1191

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, Guillou F, Sharpe RM, Verhoeven G (2004) Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc Natl Acad Sci USA* **101:** 1327–1332

Gaemers IC, van Pelt AM, van der Saag PT, Hoogerbrugge JW, Themmen AP, de Rooij DG (1997) Effect of retinoid status on the messenger ribonucleic acid expression of nuclear retinoid receptors alpha beta and gamma and retinoid X receptors alpha beta and gamma in the mouse testis. *Endocrinology* **138:** 1544–1551

Garcia-Villalba P, Jimenez-Lara AM, Aranda A (1996) Vitamin D interferes with transactivation of the growth hormone gene by thyroid hormone and retinoic acid. *Mol Cell Biol* **16:** 318–327

Ghyselinck NB, Bavik C, Sapin V, Mark M, Bonnier D, Hindelang C, Dierich A, Nilsson CB, Hakansson H, Sauvant P, Azais-Braesco V, Frasson M, Picaud S, Chambon P (1999) Cellular retinol-binding protein I is essential for vitamin A homeostasis. *EMBO J* **18:** 4903–4914

Ghyselinck NB, Vernet N, Dennefeld C, Giese N, Nau H, Chambon P, Viville S, Mark M (2006) Retinoids and spermatogenesis: lessons from mutant mice lacking the plasma retinol binding protein. *Dev Dyn* **235:** 1608–1622

Griswold MD (1995) Interactions between germ cells and sertoli cells in the testis. *Biol Reprod* **52:** 211–216

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440:** 1199–1203

Holdcraft RW, Braun RE (2004) Androgen receptor function is required in sertoli cells for the terminal differentiation of haploid spermatids. *Development* **131:** 459–467

Holstein AF, Schulze W, Davidoff M (2003) Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol* **1:** 107–123

Johnston H, Baker PJ, Abel M, Charlton HM, Jackson G, Fleming L, Kumar TR, O'Shaughnessy PJ (2004) Regulation of sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse. *Endocrinology* **145:** 318–329

Kastner P, Mark M, Ghyselinck N, Krezel W, Dupe V, Grondona JM, Chambon P (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development* **124:** 313–326

Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, Decimo D, Krezel W, Dierich A, Chambon P (1996) Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev* **10:** 80–92

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci USA* **103:** 2474–2479

Krezel W, Kastner P, Chambon P (1999) Differential expression of retinoid receptors in the adult mouse central nervous system. *Neuroscience* **89:** 1291–1300

Lecureuil C, Fontaine I, Crepieux P, Guillou F (2002) Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice. *Genesis* **33:** 114–118

Lee S, Privalsky ML (2005) Heterodimers of retinoic acid receptors and thyroid hormone receptors display unique combinatorial regulatory properties. *Mol Endocrinol* **19:** 863–878

Lindeboom F, Gillemans N, Karis A, Jaegle M, Meijer D, Grosveld F, Philipsen S (2003) Tissue-specific knockout reveals that Gata1 is not essential for sertoli cell function in the mouse. *Nucleic Acids Res* **31:** 5405–5412

Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, LeMeur M, Chambon P (1993) High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. *Proc Natl Acad Sci USA* **90:** 7225–7229

Mark M, Ghyselinck NB, Chambon P (2006) Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol* **46:** 451–480

Mascrez B, Ghyselinck NB, Watanabe M, Annicotte JS, Chambon P, Auwerx J, Mark M (2004) Ligand-dependent contribution of RXRbeta to cholesterol homeostasis in sertoli cells. *EMBO Rep* **5:** 285–290

Matt N, Dupe V, Garnier JM, Dennefeld C, Chambon P, Mark M, Ghyselinck NB (2005) Retinoic acid-dependent eye morphogenesis is orchestrated by neural crest cells. *Development* **132:** 4789–4800

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287:** 1489–1493

Morales C, Clermont Y (1993) Structural changes of the sertoli cell during the cycle of the seminiferous epithelium. In *The Sertoli Cell*, Russell LD, Griswold MD (eds) pp 306–329. Clearwater: Cache River Press

Morales C, Hugly S, Griswold MD (1987) Stage-dependent levels of specific mRNA transcripts in sertoli cells. *Biol Reprod* **36:** 1035–1046

Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P (1996) Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8 a novel retinoic acid-responsive gene. *J Cell Biol* **135:** 469–477

Parvinen M (1993) Cyclic functions of sertoli cells. In *The Sertoli Cell*, Russell LD, Griswold MD (eds) pp 331–347. Clearwater: Cache River Press

Payne C, Braun RE (2006) Glial cell line-derived neurotrophic factor maintains a POZ-itive influence on stem cells. *Proc Natl Acad Sci USA* **103:** 9751–9752

Poirier F, Robertson EJ (1993) Normal development of mice carrying a null mutation in the gene encoding the L14 S-type lectin. *Development* **119:** 1229–1236

Russell LD, Ettlin APS, Clegg RA (1990) *Histological and Histopathological Evaluation of the Testis*. Clearwater, FL, USA: Cache River Press

Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells* **24:** 1505–1511

Sharpe RM, McKinnell C, Kivlin C, Fisher JS (2003) Proliferation and functional maturation of sertoli cells and their relevance to disorders of testis function in adulthood. *Reproduction* **125:** 769–784

Timmons PM, Rigby PW, Poirier F (2002) The murine seminiferous epithelial cycle is prefigured in the sertoli cells of the embryonic testis. *Development* **129:** 635–647

Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG (1991) Proliferative activity of gonocytes sertoli cells and interstitial cells during testicular development in mice. *J Reprod Fertil* **93:** 233–243

Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, Mark M (2006) Retinoic acid metabolism and signalling pathways in the adult and developing mouse testis. *Endocrinology* **147:** 96–110

Wright WW, Smith L, Kerr C, Charron M (2003) Mice that express enzymatically inactive cathepsin L exhibit abnormal spermatogenesis. *Biol Reprod* **68:** 680–687

Yomogida K, Ohtani H, Harigae H, Ito E, Nishimune Y, Engel JD, Yamamoto M (1994) Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse sertoli cells. *Development* **120:** 1759–1766

Zabludoff SD, Charron M, DeCerbo JN, Simukova N, Wright WW (2001) Male germ cells regulate transcription of the cathepsin l gene by rat sertoli cells. *Endocrinology* **142:** 2318–2327

Zhao GQ, Deng K, Labosky PA, Liaw L, Hogan BL (1996) The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev* **10:** 1657–1669

Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl* **23:** 870–881