Expression of retinoic acid receptor alpha in the germline is essential for proper cellular association and spermiogenesis during spermatogenesis

Sanny S. W. Chung[1,2,3], Xiangyuan Wang[1] and Debra J. Wolgemuth[1,2,3,4],*

Signaling through vitamin A metabolites is indispensable for spermatogenesis, and disruption of retinoic acid receptor alpha (RARα) function resulted in male sterility and aberrant spermatogenesis, which resembled vitamin A deficiency. Here we investigated the lineage- and cell-specific role of RARα-mediated signaling during spermatogenesis using germ-cell transplantation and genetically manipulated mouse models. We demonstrated that RARα-deficient germ-cell stem cells were able to repopulate germ-cell-depleted wild-type testes and initiate spermatogenesis; however, improper cellular associations and abnormal sperm formation were observed. We further generated RARα-deficient mice that expressed RARα-EGFP fusion protein uniquely in haploid germ cells. Strikingly, spermatid orientation, alignment and release, as well as sperm morphology, were normal and there was a partial rescue of sterility. These data provide the first direct evidence for a distinct requirement of RARα-mediated retinoid signaling specifically in germ cells.

KEY WORDS: Retinoid signaling, Spermiogenesis, Germ-cell transplantation, Lineage-specific function, Mouse

INTRODUCTION

Mammalian spermatogenesis is a highly organized process with three distinct phases: spermatogonial proliferation, meiosis of spermatocytes and differentiation of haploid spermatids during spermiogenesis. During spermiogenesis, there is extensive chromatin remodeling and compaction, resulting in the highly condensed nucleus of spermatozoa. Spermatogenesis culminates in spermiation, with release of spermatozoa into the tubular lumen. This differentiation is controlled in part by Sertoli cells, which physically interact with germ cells via cellular connections in the seminiferous tubules (Cheng and Mruk, 2002). The timing of spermatogenesis is under rigid control, resulting in characteristic association of different spermatogenic cell types, known as the seminiferous epithelial cycle (Russell et al., 1990). Genetic control of this timing appears to be intrinsic to the germ cells – rat-specific timing was observed in rat germ cells transplanted into mouse testes (Franca et al., 1998) – but the underlying mechanisms remain unknown. This tightly controlled cycle can, however, be altered by changes in retinoid signaling (Chung and Wolgemuth, 2004; de Rooij et al., 1994; Ismail et al., 1990; Morales and Griswold, 1987).

Since the 1920s, vitamin A (dietary retinol) has been recognized as essential for normal spermatogenesis (Howell et al., 1963; Wolbach and Howe, 1925; Chung and Wolgemuth, 2004). The abnormalities that occur at specific stages of spermatogenesis as a result of vitamin A deficiency (VAD) have been extensively studied, particularly in the rat (Chung and Wolgemuth, 2004; de Rooij et al., 1994; Eskild and Hansson, 1994; Griswold et al., 1989). Although the morphological changes in spermatogenesis upon VAD have been well documented, the molecular mechanisms underlying these

changes are still largely unknown and are likely to be complex. The role during spermatogenesis of several genes involved in retinoid synthesis, transport, nuclear and cytoplasmic binding, and degradation has been addressed by gene-targeting strategies (Chung and Wolgemuth, 2004; Livera et al., 2002). However, only the retinoic acid receptor alpha (RARα) knockout model exhibited defects in spermatogenesis that closely resembled VAD (Lufkin et al., 1993).

To understand the molecular and mechanistic basis for the aberrant spermatogenesis in $Rara^{-/-}$ mice, we undertook a detailed analysis of the morphological properties and the time of onset of the abnormalities (Chung et al., 2004; Chung et al., 2005; Chung and Wolgemuth, 2004; Wolgemuth and Chung, 2007). These observations revealed prominent abnormalities in spermiogenesis (Chung et al., 2004; Chung et al., 2005), particularly in spermiation, that were similar to those in VAD testes, suggesting that mechanisms underlying spermiogenesis and completion of spermiation are extremely sensitive to change in the status of retinoid signaling and must involve, at least in part, RARα-mediated signaling pathways. These studies further revealed the crucial role of RARα in other stages of germ-cell differentiation, specifically the establishment of normal progression of spermatogenesis and the subsequent formation of correct cellular associations. However, it remained unclear whether RARα signaling is required in germ-cell or somatic-cell lineages, or both.

There is an extensive body of literature documenting the expression of $Rara$ transcripts and RARα protein in both germ cells and somatic cells [Chung and Wolgemuth (Chung and Wolgemuth, 2004) and references therein]. In particular, RARα was shown to be important in both germ cells and Sertoli cells for re-initiation of spermatogenesis (Akmal et al., 1998; de Rooij et al., 1994; Morales and Griswold, 1987; van Pelt et al., 1992). However, a recent study by Vernet et al. (Vernet et al., 2006b) reported that although $Rara$ transcripts were found in both germ cells and Sertoli cells, RARα protein was detected only in Sertoli cells. Follow-up studies by the same group in which $Rara$ was ablated only in Sertoli cells ($Rara^{Ser-/-}$) revealed testis degeneration and delayed

[1]Department of Genetics and Development, [2]Department of Obstetrics and Gynecology, [3]The Institute of Human Nutrition and [4]The Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, NY 10032, USA.

*Author for correspondence (e-mail: djw3@columbia.edu)

spermatogonial expression of the RA-responsive gene, *Stra8* (Vernet et al., 2006a). However, the duration of the cycle of the seminiferous epithelium was reported not to be affected, and the typical 12 stages of the cycle with apparently normal cellular associations were seen (Vernet et al., 2006a). With time, however, most of the tubules of *Rara*$^{Ser-/-}$ mutants contained only Sertoli cells, indicating the essential requirement of RARα in Sertoli cells for maintenance of the germ-cell epithelium (Vernet et al., 2006a). This is in contrast to the aberrant cellular associations in *Rara*$^{-/-}$ testicular tubules, which lack RARα in both germ and somatic cells, and suggested a unique requirement of RARα-mediated signaling in germ cells for normal progression of spermatogenic development (Chung et al., 2004).

To test the hypothesis that RARα is required in both germ-cell and somatic-cell lineages, we examined the capability of donor *Rara*$^{-/-}$ spermatogonial stem cells to colonize and differentiate in germ-cell-depleted wild-type testes, and vice versa. Concomitantly, we asked whether targeted expression of RARα in haploid germ cells can rescue some or all of the testicular abnormalities in *Rara*$^{-/-}$ mice. This study is the first demonstration of a distinct requirement for RARα in the germline for proper cellular associations and further provides the first direct evidence of a crucial role of RARα in spermatid development, alignment and release.

MATERIALS AND METHODS
Source of animals and tissues
All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Columbia University Medical Center. Testes were dissected from animals perfused with phosphate buffered saline (PBS) and then with 4% paraformaldehyde (PFA) in PBS or with Bouin's fixative, overnight at 4°C. Testes were either embedded in paraffin or frozen in liquid nitrogen as described previously (Chung et al., 2004).

Production of mice lacking RARα uniquely in the germinal or somatic lineage by germ-cell transplantation
Male transgenic mice that carry the β-actin promoter driving enhanced green fluorescent protein (EGFP) (Okabe et al., 1997) (C57BL/6-Tg(ACTbEGFP)1Osb/J, green mice; The Jackson Labs; designated *Actb-EGFP*) were crossed with *Rara*$^{-/-}$ females. The resulting heterozygous *Rara*$^{+/-}$; *Actb-EGFP*$^+$ progeny were examined for expression of EGFP as assessed by green fluorescence under ultraviolet light (365 nm) and bred with *Rara*$^{-/-}$; *Actb-EGFP*$^+$ females. Males that were *Rara*$^{-/-}$; *Actb-EGFP*$^+$ or *Rara*$^{+/+}$; *Actb-EGFP*$^+$ were used for transplantation experiments. Germ-cell transplantation was performed as previously described (Brinster and Avarbock, 1994; Costoya et al., 2004; Ogawa et al., 1997). Briefly, suspensions of donor total germ cells were obtained from 5 to 60 day-old mice, as described previously (Wolgemuth et al., 1985; Chalmel et al., 2007). The cell pellet was suspended in Dulbecco's modified Eagle's Medium at 5×10^5 cells/10 μl. Approximately 10-30 μl of the suspension containing Trypan Blue were transplanted into the rete testes of NCr Nude Outbred (nu/nu; NCRNU-M; Taconic) mice that had been treated with busulfan (40 mg/kg) by intraperitoneal injection at 4-6 weeks of age. Busulfan-treated recipient testes were shown by histological assessment to be virtually devoid of endogenous germ cells at the time of transplantation, ~4-6 weeks after busulfan treatment.

The *Rara*$^{-/-}$ mice had been routinely maintained on a mixed 129/C57BL/6 background. To use *Rara*$^{-/-}$ testes as recipients for *Rara*$^{+/+}$; *Actb-EGFP*+ germ cells on a C57BL/6 background without immunological rejection, *Rara*$^{-/-}$ mice were backcrossed onto C57BL/6 mice and germ-cell transplantation was performed as above.

Analysis of colonization of recipient mice
The extent of colonization was assessed as described previously (Brinster and Avarbock, 1994; Costoya et al., 2004; Ogawa et al., 1997). Briefly, after 3-5 months, colonies of donor-cell-derived spermatogenesis were easily identified in recipient testes as EGFP$^+$ tubules. Testes were fixed overnight with 4% PFA, embedded in OCT compound (Tissue-Tek, CA, USA), sectioned, covered with Dako Glycergel mounting medium (Dako North America, CA, USA), and viewed on a Nikon Eclipse 800 photomicroscope under fluorescence. To quantify the percentage of tubules repopulated by the donor, we analyzed three 8-μm histological sections chosen randomly from the middle of the longitudinal axis of the testes, each separated by more than 200 μm. For each section, at least 150 tubules were evaluated and quantified. The sections were also processed for Hematoxylin staining and immunohistochemistry. To evaluate specific cell types, at least 40 repopulated tubules of each testis from five different recipient animals were counted per group as described previously (Chung et al., 2005). Significant differences were assessed by statistical analysis using Student's paired *t*-test using GraphPad Analysis software.

Generation of *Prm1-Rara/EGFP* transgenic mice and crossing onto *Rara*$^{-/-}$ mice
A cDNA of the mouse *Rara* transcript containing the entire coding sequence (Zelent et al., 1989) (provided by Cathy Mendelsohn, Columbia University) was fused in-frame to EGFP in pEGFP-N1 vector (Clontech, CA, USA) under the cytomegalovirus (CMV) promoter. For expression in round spermatids, this *CMV-Rara/EGFP* construct was substituted CMV with a 652-bp regulatory element for mouse protamine 1 (*Prm1*) gene (provided by Stephen O'Gorman, Case Western Reserve University). This promoter had been used previously and does not produce any testicular abnormalities (O'Gorman et al., 1997). The *Prm1*-driven *Rara/EGFP* constructs (Fig. 3A) have a deletion of 133 bp of 3′ UTR of *Prm1*, which allowed translation earlier than the endogenous *Prm1* mRNA (Braun et al., 1989; Fajardo et al., 1997). Transgenic mice (*Prm1-Rara/EGFP*) were generated following our laboratory's standard procedures (Liao et al., 2001). Transgenic mice were genotyped using two primers, 5′-GGTCGCCACCATGGTGAGCAAGGG-3′, and 5′-TACTTGTACAGCTCGTCCATGCCG-3′, corresponding to EGFP in pEGFP-N1 vector (Sigma-Aldrich, MO, USA), yielding a 729-bp product. The PCR conditions were 10 minutes at 94°C, then 35 cycles of 1 minute each at 94°C, 58°C and 72°C. Expression of EGFP was examined with excitation light of 488 nm and confirmed by immunohistochemistry using GFP antibody (Abcam Inc, MA, USA). F1 progeny were obtained by breeding founder animals with B6CBAF1/J mice. *Rara*$^{-/-}$ mice were genotyped as described (Lufkin et al., 1993) using three primers, 5′-GCC-TTCTATCGCCTTCTTGACGAGTTCTTC-3′, 5′-TGTGCCCTTCCCT-CCATCTTCCTT A-3′ and 5′-TCCGACTTGCGACTCCCTCTACTCA-3′ (Invitrogen, CA, USA), using the same PCR conditions.

Prm1-Rara/EGFP transgenic males were intercrossed with *Rara*$^{-/-}$ females to generate *Rara*$^{+/-}$; *Prm1-Rara/EGFP*$^+$ males and *Rara*$^{+/-}$; *Prm1-Rara/EGFP*$^+$ females. These animals were then intercrossed to generate *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ males. The fertility status of these *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ males was also examined.

Immunohistochemistry
Frozen sections from perfused, fixed testes, post-fixed in acetone, were immunostained as previously described (Chung et al., 2004). Rabbit polyclonal antibodies to EGFP (Abcam, MA, USA) were diluted 1:100 in 1×PBS and anti-Brdt antibodies (Shang et al., 2007) at a 1:1000 dilution. Stained sections were examined by bright-field microscopy. To evaluate specific cell types, only clearly stained cells were considered to be positive and only round-shaped tubules were assessed. At least 100 tubules of each testis from three different animals were counted per group as described previously (Chung et al., 2005). Significant differences were assessed by statistical analysis using Student's paired *t*-test using GraphPad Analysis software.

Assessment of fertility and fecundity
Mating studies were carried out to assess fertility and fecundity of *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ male mice as described by others (Jeffs et al., 2001). Eight-week-old males were housed individually with two or three 6-week-old fertile B6CBAF1/J wild-type females. Mounting behavior was observed, and females were examined the next morning for copulatory plugs. Fertility was evaluated as the average number of litters produced by each pair.

Fecundity was determined as the average number of live pups per litter and the average number of pups weaned (21 days old). Each breeding pair remained together for >6 months and the number of progeny was recorded.

One step Eosin-Nigrosin staining of spermatozoa

The Eosin-Nigrosin staining solution contained 0.67% Eosin Y yellow (Fisher Scientific, PA, USA) and 10% Nigrosin (Sigma-Aldrich, MO, USA) and was used to stain spermatozoa as described previously (Bjorndahl et al., 2003). Sperm morphology was examined by bright-field microscopy.

RESULTS

Rara$^{-/-}$ spermatogonial stem cells were able to colonize and initiate spermatogenesis in germ-cell-depleted *Rara*$^{+/+}$ tubules

To test the hypothesis that RARα has distinctive roles in both germ-cell and somatic-cell lineages, suspensions of germ cells expressing EGFP under the β-actin promoter and comprised of various stages of differentiating spermatogenic cells, including stem cells from *Rara*$^{+/+}$ or *Rara*$^{-/-}$ mice (*Rara*$^{+/+}$; *Actb-EGFP*$^{+}$ and *Rara*$^{-/-}$; *Actb-EGFP*$^{+}$, respectively) were transplanted into testes of immunodeficient nude mice (*Rara*$^{+/+}$) that had been chemically depleted of germ cells. Six months after transplantation, there was successful colonization of tubules by both *Rara*$^{+/+}$; *Actb-EGFP*$^{+}$ (Fig. 1A) and *Rara*$^{-/-}$; *Actb-EGFP*$^{+}$ (Fig. 1B-D) spermatogonial stem cells, as evidenced by the presence of differentiating fluorescent germ cells. The fluorescence was readily detected in spermatogonia and spermatocytes and gradually decreased after meiosis, as had been reported by others using the *Actb-EGFP*$^{+}$ transgenic line (Kanatsu-Shinohara et al., 2003; Ohta et al., 2000). *Rara*$^{-/-}$ germ cells were therefore able to proliferate and begin to differentiate in the presence of a wild-type somatic compartment.

Histological evaluation (Fig. 1E-F) confirmed the presence of RARα-deficient spermatogenic cells that had progressed as far as the elongated spermatid stages (arrows in Fig. 1E,F). However, the epididymal spermatozoa exhibited abnormal morphologies, with blunted heads (Fig. 1H), in contrast to the normal sickle-shaped spermatozoa in mice transplanted with *Rara*$^{+/+}$; *Actb-EGFP*$^{+}$ cells (Fig. 1G). The number of repopulated tubules was similar using *Rara*$^{-/-}$ or *Rara*$^{+/+}$ donor cells (Fig. 1I). Thus, *Rara*$^{-/-}$ germ cells can colonize the recipient testes and initiate spermatogenesis, but full restoration of spermatogenesis may require RARα function in germ cells during later stages of spermatogenic differentiation.

Rara$^{-/-}$ germ cells undergo spermatogenesis but with improper cellular associations

We next examined the cellular associations in the repopulated tubules in greater detail. Tubules with endogenous *Rara*$^{+/+}$; *Actb-EGFP*$^{-}$ (upper two tubules in Fig. 2A,B, and 2C) or transplanted *Rara*$^{+/+}$; *Actb-EGFP*$^{+}$ germ cells (data not shown) exhibited normal cellular associations. By contrast, although *Rara*$^{-/-}$; *Actb-EGFP*$^{+}$ germ cells repopulated and re-initiated spermatogenesis (bottom two tubules in Fig. 2A,B, and 2D), various abnormalities were observed. There was aberrant nuclear condensation among spermatids within individual tubules, resulting in atypical steps of spermatids being present within a single tubule (arrows, left insert, Fig. 2D). Furthermore, there were missing or decreased numbers of the predicted cell types in repopulated tubules (Fig. 2G). For example, the upper and lower tubules in Fig. 2D contained 69 and 58 round spermatids (dashed green line), respectively, compared with ~146 round spermatids in the control (bottom tubule, Fig. 2C). Some were abnormally shaped and their nucleoli were diffusely stained (upper insert in Fig. 2D). Apparently normal numbers of pachytene spermatocytes were seen
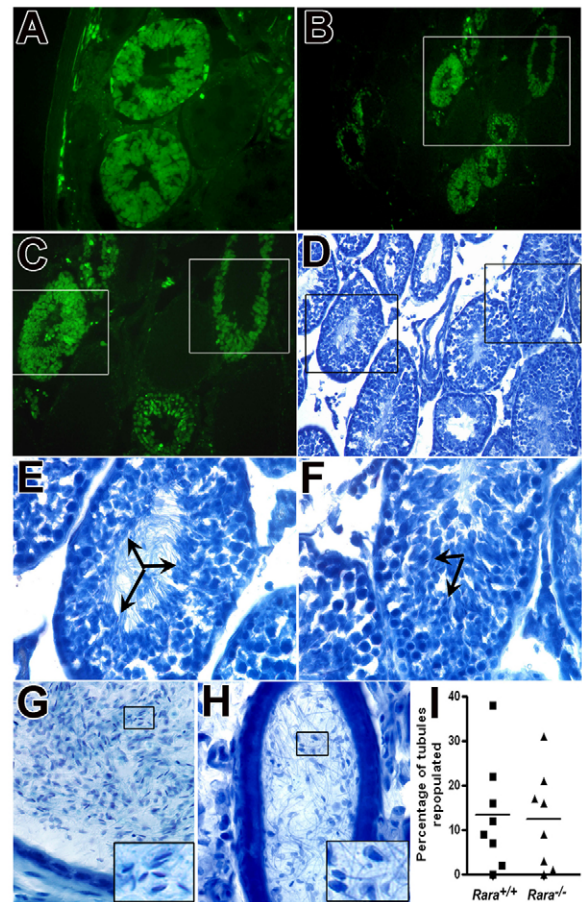
Fig. 1. *Rara*$^{-/-}$ spermatogonial stem cells repopulated and initiated spermatogenesis in germ-cell-depleted recipient testis. (**A-F**) Spermatogonial stem cells from *Rara*$^{+/+}$; *Actb-EGFP*$^{+}$ (A) and *Rara*$^{-/-}$; *Actb-EGFP*$^{+}$ (B-F) mice were transplanted into the tubules of busulfan-treated nude mice. Representative sections of the testes at 6 months after transplantation viewed under a fluorescent microscope at low (B) and higher (C) magnification showed repopulated green-fluorescing *Rara*$^{-/-}$ cells in some but not all tubules, indicating the successful colonization of transplanted cells. The adjacent serial section, stained with Hematoxylin, is shown at higher magnification (D). C represents a higher magnification of the insert in B; E and F show higher magnification of the inserts in C and D. Arrows in E and F indicate elongated spermatids. (**G,H**) Abnormal sperm heads were observed in the epididymis after transplantation (H) compared with normal sickle-shaped spermatozoa in control epididymis (G). Magnification: B, ×10; A,C,D, ×20; E-H, ×60. (**I**) Graph showing the percentages of tubules in recipient testis with restored spermatogenesis. Individual counts from each testis are indicated with squares and triangles; the mean of each data set is plotted with a horizontal bar. The number of repopulated tubules from the *Rara*$^{-/-}$ transplantation was similar to that from the *Rara*$^{+/+}$.

in tubules regardless of the source of the repopulating germ cells (dashed red line, Fig. 2D) with 66 and 58 pachytene spermatocytes in the upper and lower tubules, respectively, compared with controls (56, bottom tubule, Fig. 2C; and 64, upper right tubule, Fig. 2F). Interestingly, in another repopulated *Rara*$^{-/-}$; *Actb-EGFP*$^{+}$ spermatogenic tubule (Fig. 2E), there was almost an entire layer of pachytene spermatocytes missing (5, left tubule and insert in Fig. 2F), resembling the characteristic phenotypes observed in *Rara*$^{-/-}$ testes
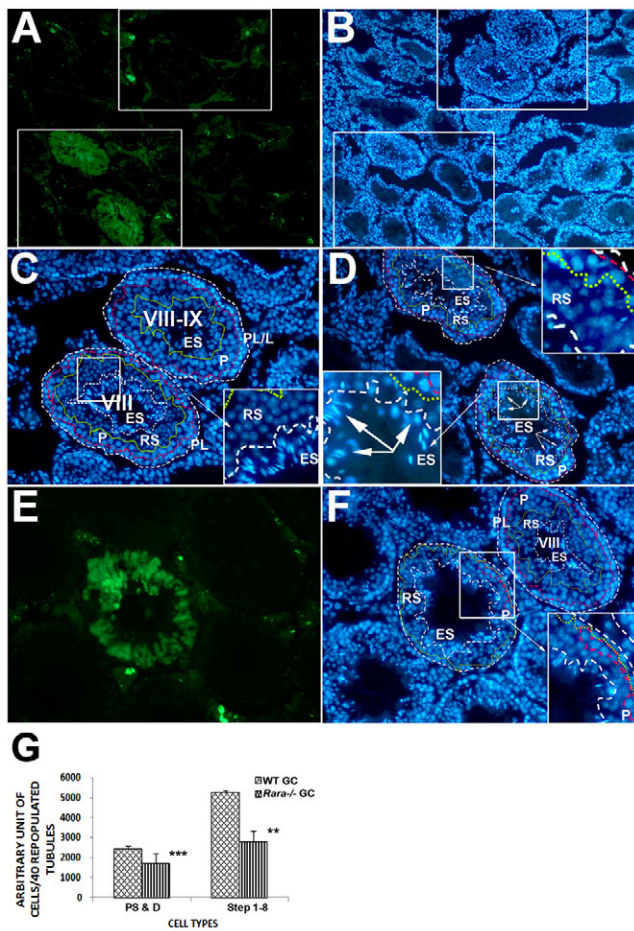
Fig. 2. *Rara*$^{-/-}$ **germ cells supported by** *Rara*$^{+/+}$ **somatic cells exhibit improper cellular associations.** (**A-F**) Representative fluorescent sections of testes at 5 months after transplantation showing repopulated green-fluorescing *Rara*$^{-/-}$ germ cells in some but not all tubules, as well as the repopulated endogenous non-fluorescing *Rara*$^{+/+}$ germ cells (A,E). The same sections, counterstained with DAPI, are shown (B,F, respectively). C and D are higher magnifications of the tubules in B, illustrating various spermatogenic cell layers. The outer dashed white line encircles the outline of seminiferous tubules, spermatogonia, pre-leptotene and/or leptotene spermatocytes; the red dashed line encircles pachytene spermatocytes; the green dashed line encircles round spermatids; and the inner dashed white line encircles elongated spermatids. (C) Stage VIII tubules containing four layers of cells, with an outer layer of pre-leptotene spermatocytes, pachytene spermatocytes, step 8 round spermatids and step 16 elongated spermatids at the innermost layer. The round spermatids contained characteristic large and densely stained nucleoli (insert in C). An adjacent tubule (at the end of stage VIII and the beginning of stage IX) contained three layers of cells: pre-leptotene/leptotene spermatocytes at the basal lamina, pachytene spermatocytes in the middle, and step 8-9 spermatids at the innermost layer. Roman numerals in this figure and in Figs 3 to 6 indicate the stage of the tubules (Russell et al., 1990). (**G**) Spermatogenic cell distribution in repopulated tubules transplanted with wild-type germ cells (WT GC) and *Rara*$^{-/-}$ germ cells (*Rara*$^{-/-}$GC). The total number of various cell types [pachytene spermatocytes and diplotene spermatocytes (PS and D); step 1-8 round spermatids (Step 1-8)] per 40 repopulated tubules were counted. Error bars represent the mean±s.d. of the counts. ***, *P*<0.01 and **, *P*<0.05. Magnification: A,B, ×20; C-F, ×40. ES, elongated spermatids; L, leptotene spermatocytes; P, pachytene spermatocytes; PL, pre-leptotene spermatocytes; PL/L, pre-leptotene/leptotene spermatocytes; RS, round spermatids.

(Chung et al., 2004). The adjacent tubule that contained only non-fluorescing germ cells (presumably endogenous *Rara*$^{+/+}$; *Actb-EGFP*$^-$ germ cells) (upper right tubule in Fig. 2E) displayed the proper four layers of germ cells characteristic of stage VIII tubules (Fig. 2F). Thus, tubules populated with *Rara*$^{-/-}$; *Actb-EGFP*$^+$ spermatogonial stem cells seemed to undergo spermatogenesis but with abnormal cellular associations.

Expression of *Prm1-Rara* in spermatids in *Rara*$^{-/-}$;*Prm1-Rara/EGFP*$^+$ mice can restore fertility

We then extended our analysis to ask if RARα has distinct functions in different stages of development within the germline lineage, in particular in differentiating spermatids. Specifically, we generated transgenic mice expressing an RARα-EGFP fusion protein uniquely in spermatids (Fig. 3A). Three independent lines of mice carrying *Prm1-Rara/EGFP* transgenes were established. Two lines that had been stably maintained for >3 years and characterized in detail were then used in the 'rescue' experiments described below. The *Prm1-Rara/EGFP* transgenic males appear fully fertile, so there does not seem to be an adverse affect of overproducing RARα/EGFP.

Green fluorescence was readily detected in intact testes of the *Prm1-Rara/EGFP* transgenic mice (Fig. 3Ba) but not in non-transgenics (Fig. 3Bb). The cellular specificity of expression of the *Prm1-Rara/EGFP* transgene was confirmed by histological sections that revealed green-fluorescing spermatids at the correct stages of differentiation (Fig. 3Ba,c). Non-specific autofluorescence of the interstitial regions was detected in both transgenic and non-transgenic testes (Fig. 3Bc,d). Immunohistochemical staining confirmed its expression in step 7 round spermatids at stage VII (Fig. 3Bf), in steps 9-12 elongating spermatids at stage IX-XII (Fig. 3Be-g) and in steps 13-16 elongated spermatids at stage I-VIII (Fig. 3Be-g), but not in wild-type mouse testes (Fig. 3Bh). As expected, no expression was detected in spermatogonia, spermatocytes and Sertoli cells, or in the interstitial compartment (Fig. 3Be-g). This also confirmed that the autofluorescence seen in Fig. 3Bc,d was indeed non-specific.

We next asked whether expressing this transgene in haploid spermatids of *Rara*$^{-/-}$ mice could rescue spermiogenesis, and yield normal spermatozoa and a restoration of fertility. We used *Rara*$^{+/+}$; *Prm1-Rara/EGFP*$^+$ male mice from the line that showed higher expression of the transgene in haploid spermatids for intercrossing with female *Rara*$^{-/-}$ mice to produce *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ mice. We identified *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ mice that could generate progeny (*n*=6 out of 40) (Table 1). The *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ rescued males gave birth to normal-appearing pups, but had smaller average litter sizes, ranging from 2.0 to 5.5 (Table 1) compared with the litter sizes of transgenic (*Rara*$^{+/+}$; *Prm1-Rara/EGFP*$^+$) mice of 9.5±1.45, *n*=12, and control (*Rara*$^{+/+}$; *Prm1-Rara/EGFP*$^-$) mice of 9.8±1.40, *n*=12. The gender distribution of the offspring of the rescued males appeared to be normal; for example, with four males and three females in a litter of seven (Table 1), and these progeny grew and bred normally.

Characterization of spermatogenesis in *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ testes

We next examined the extent and progression of spermatogenesis in these mice in detail. Remarkably, *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^+$ tubules from the rescued mice at 4 months of age were similar in size and morphology to wild-type tubules, containing the typical three to four layers of spermatogenic cells (Fig. 4E versus 4A). Notably, an almost complete rescue of spermiogenesis and sperm formation was observed (Fig. 4E,F, respectively), with normal, sickle-shaped spermatozoa in the epididymides (Fig. 4F versus 4B).
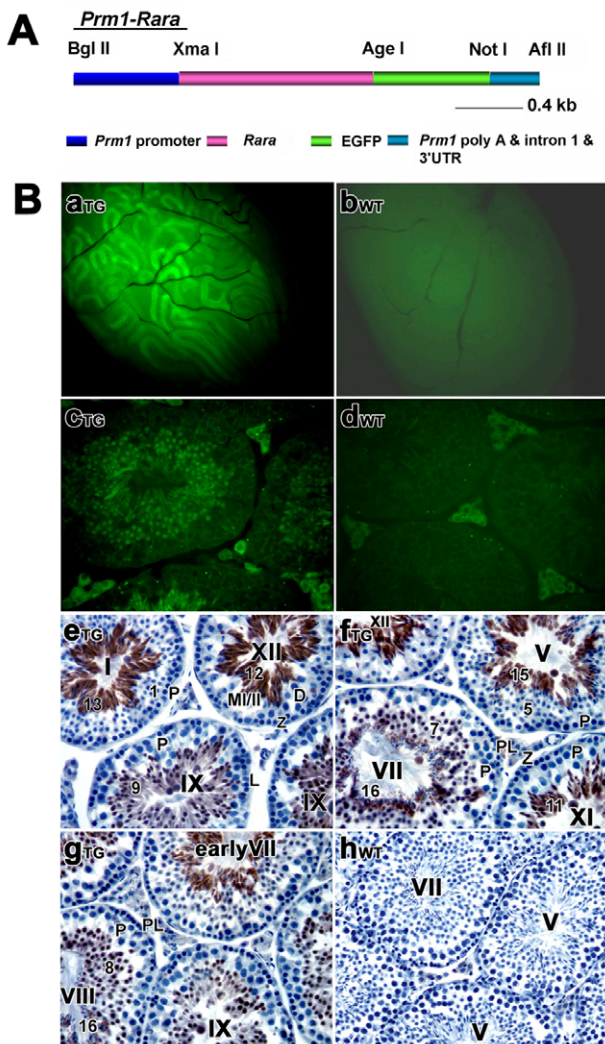
Fig. 3. Transgenic model to overexpress *RARα/EGFP* in round and elongated spermatids. (**A**) Diagram of the *Prm1-Rara/EGFP* transgene. *Prm1*, a spermatid-specific promoter, was used to drive expression of an *Rara* cDNA fused in-frame to EGFP coding sequences (construct: *Prm1-Rara/EGFP-Prm1 poly A* and intron 1). (**B**) Expression of EGFP in the testis of a *Prm1-Rara/EGFP* transgenic mouse at 6 weeks of age. Intact testes of transgenic and wild-type mice were observed under fluorescent light at 6 weeks (a,b, respectively). Representative sections examined under a fluorescent microscope (c,d) revealed that green-fluorescing cells were detected in some but not all tubules, suggesting the transgene expression is specific to particular stages of spermatid differentiation (a,c) and that there is no expression in the wild-type testis (b,d). Immunohistochemical detection of EGFP on histological sections of 6-week-old testes from transgenic mice (e-g). EGFP was detected in nuclei of round spermatids at stage VII (f), elongating spermatids at stage IX-XII (e-g), and elongated spermatids at stage I-VIII (e-g) of adult transgenic but not control (h) testes. Magnification: c-h, ×40. Arabic numerals indicate the step of spermatids shown. D, diplotene spermatocytes; L, leptotene spermatocytes; P, pachytene spermatocytes; PL, pre-leptotene spermatocytes; Z, zygotene spermatocytes.

By contrast, testes of non-rescued *Rara*^{−/−}; *Prm1-Rara/EGFP*[+] mice were morphologically similar to *Rara*^{−/−} testis (asterisks in Fig. 4G,C, respectively). Layers of spermatogenic cells were missing in many tubules, and in some tubules only pachytene spermatocytes were found (middle left tubule, Fig. 4G), similar to *Rara*^{−/−} testes

(right tubule, Fig. 4C). Furthermore, only round/oval, abnormally shaped, undifferentiated and degenerated spermatids were seen in the epididymides (inserts in Fig. 4H,D, respectively). Weak expression of the transgene was found in these mice as examined under fluorescent microcopy (data not shown), which could explain in part the failure to rescue.

We further assessed quantitatively the variation in the number of pachytene spermatocytes and round spermatids (steps 1-8) using specific markers and periodic acid Schiff (PAS) staining. Stained pachytene spermatocytes and round spermatids can be easily distinguished by their size. PAS staining of spermatids to clearly visualize acrosome caps was used to further distinguish spermatids (data not shown). Brdt, a testis-specific member of the BET subfamily of bromodomain-motif-containing proteins, was used as a marker for pachytene spermatocytes and round spermatids (Shang et al., 2007). Immunostaining revealed the expected pattern of Brdt expression in both rescued and non-rescued *Rara*^{−/−}; *Prm1-Rara/EGFP*[+] testes (Fig. 4I,J). We observed fewer pachytene and diplotene spermatocytes (~28%) as well as round spermatids (~36%) in the non-rescued tubules, compared with rescued testes (Fig. 4K).

Successful spermatid orientation, alignment and release in rescued males
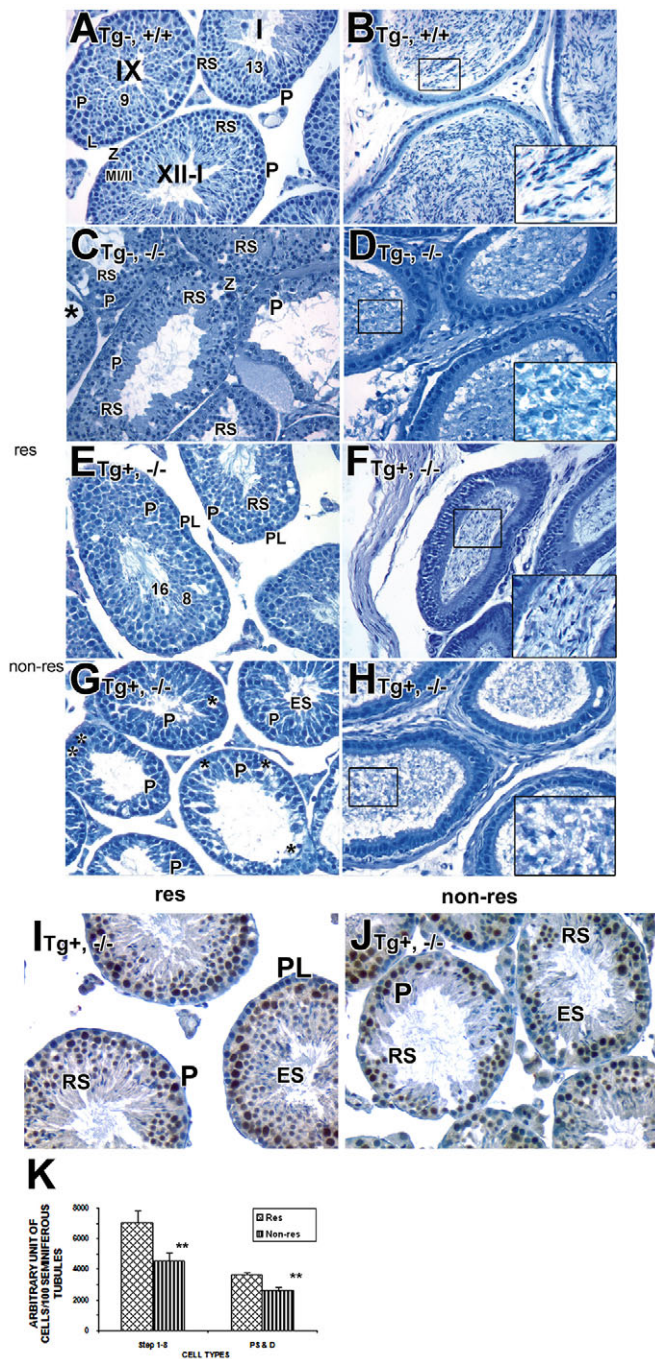
As mentioned before, spermatid alignment and release have been reported to be abnormal in *Rara*^{−/−} testes (Chung et al., 2004; Chung et al., 2005). Both functions were restored in rescued *Rara*^{−/−}; *Prm1-Rara/EGFP*[+] mice: step 16 spermatids aligned properly at the tubular lumen of stage VIII tubules (Fig. 5A), and at stage IX almost all step 16 spermatids had been released and were only rarely found in the seminiferous tubules (Fig. 5B). The acrosomes of step 9 spermatids now oriented properly, facing towards the basal aspect of the Sertoli cells (Fig. 5B). Furthermore, spermatozoa with normal sickle-shaped heads were noted in the epididymides (Fig. 4F).

Morphology of wild-type, *Rara*^{−/−} and rescued spermatozoa

Spermatozoa from the cauda epididymides of *Rara*^{+/+} mice exhibited the characteristic hooked-head morphology (98.2±2.02%; *n*=100 spermatozoa/animal) (Fig. 5C), whereas almost all *Rara*^{−/−} spermatozoa examined had aberrantly shaped heads and exhibited a tapered, round or ovoid shape (97±1.56%; *n*=100 spermatozoa/animal) (Fig. 5D-F). Frequently, the normally sharp apex was bent or blunted. In some instances, the mid-piece of the sperm tail, which contains the mitochondrial sheath, was noticeably thinned (arrow in Fig. 5F) and a number of spermatozoa had their tails coiled around their nuclei (Fig. 5F). Moreover, the residual spermatozoa appeared immotile; only ~2% displayed a sluggish progression or non-progressive motility. Thus, *Rara*^{−/−} males suffered from oligo-astheno-teratozoospermia (low sperm number, low sperm motility and abnormal sperm morphology) resulting in sterility. By contrast, spermatozoa from the rescued males had normal-appearing sickle-shaped heads with apical hooks, similar to wild type (90±3.02%; *n*=100 spermatozoa/animal) (Fig. 5G versus 5C). This suggested that the expression of RARα in round spermatids of *Rara*^{−/−} mice was able to support normal differentiation of spermatozoa, and hence capable of restoring fertility.

Sloughing of spermatogenic cell layers in the testes of rescued mice with time

Over the 6-month mating period, the rescued males lost fertility. Histological evaluation of one of the testes of a rescued but now sterile *Rara*^{−/−}; *Prm1-Rara/EGFP*[+] male at 8.5 months of age

Fig. 4. Expression of *RARα* in round and elongated spermatids can rescue spermiogenesis in *Rara*^−/−^ testes. (A-H) Histological sections of testes from wild-type (A), *Rara*^−/−^ (C), rescued *Rara*^−/−^; *Prm1-Rara/EGFP* (E) and non-rescued *Rara*^−/−^; *Prm1-Rara/EGFP* (G) mice at 4 months of age are shown with their corresponding epididymides (B,D,F,H, respectively). Inserts in B, D, F and H show higher magnifications. (**I,J**) Immunostaining of rescued *Rara*^−/−^; *Prm1-Rara/EGFP* (I) and non-rescued *Rara*^−/−^; *Prm1-Rara/EGFP* (J) testes are shown using Brdt (I,J) antibodies. Asterisks mark vacuoles in the tubules. (**K**) Spermatogenic cell distribution in rescued *Rara*^−/−^; *Prm1-Rara/EGFP* and non-rescued *Rara*^−/−^; *Prm1-Rara/EGFP* mice. The total number of various cell types [step 1-8 round spermatids (Step 1-8); pachytene spermatocytes and diplotene spermatocytes (PS and D)] per 100 seminiferous tubules was counted. Error bars represent the mean±s.d. of the counts. **, $P<0.05$. Arabic numerals indicate the step of spermatids shown. Magnification: A-J, ×40. ES, elongated spermatids; L, leptotene spermatocytes; MI/II, meiosis I/II; non-res, non-rescued mice; P, pachytene spermatocytes; PL, pre-leptotene spermatocytes; res, rescued mice; RS, round spermatids; Z, zygotene spermatocytes.

age (Fig. 6C) and in older *Rara*^−/−^ mice reported previously (Lufkin et al., 1993). Normal-looking spermatozoa were almost never detected in the corresponding epididymides of the previously rescued but now infertile mice (Fig. 6F).
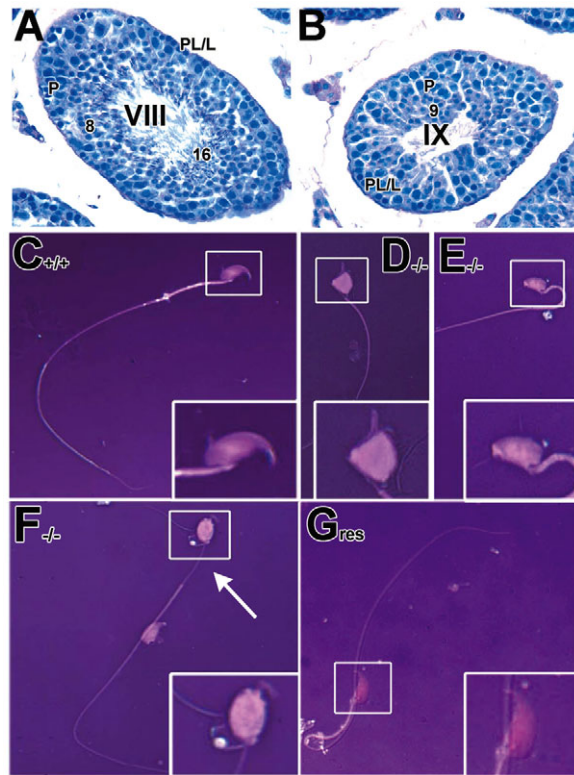
The remaining testis from the same rescued male was removed at 15.5 months of age. Severe loss of germ-cell layers and germ-cell-depleted, Sertoli-cell-only tubules were found (Fig. 6G), and, as expected, no spermatozoa were found in the corresponding epididymis (Fig. 6H). Sloughing of spermatogenic cell layers with time was consistently found in the four rescued males examined. To determine whether the infertility in previously rescued but now infertile mice was due to an unexpected loss of RARα expression in haploid spermatids, the testes were immunostained for EGFP. EGFP was consistently found in any remaining round and elongated spermatids (Fig. 6I,J). Together, these observations suggested that the fertility loss in the older rescued males is due to severe sloughing of germ-cell layers.

RARα in the somatic-cell lineage is essential for normal donor germ-cell stem cells to colonize the *Rara*^−/−^ germ-cell-depleted recipient seminiferous tubules

We next conducted reciprocal germ-cell transplantation experiments in which wild-type RARα (*Rara*^+/+^; *Actb-EGFP*^+^) germ cells were introduced into *Rara*^−/−^ recipient tubules. To use *Rara*^−/−^ testes as recipients for *Rara*^+/+^; *Actb-EGFP*^+^ germ cells on a C57BL/6 background without immunological rejection, *Rara*^−/−^ mice were backcrossed with C57BL/6 mice to obtain pure backgrounds (Crusio, 2004; Wolfer et al., 2002). *Rara*^−/−^ mutant mice at the eighth generation (99.22% C57Bl/6J, Charles River Laboratories, MA,

revealed that cell layers were detached from the tubules, perhaps resulting in the vacuolar spaces detected (asterisks in Fig. 6E). This resembled the loss of spermatogenic cells and presence of vacuoles observed in tubules of the non-rescued males at the corresponding

Table 1. The progeny obtained from spermatid-specific rescue of fertility in *Rara*^−/−^; *Prm1-Rara/EGFP*^+^ males

| Fertile males (Tg+, −/−)	Time to analysis (days)	Days to first progeny	Number of litters	Gender of offspring (males, females)	Average litter size
#1183	209	113	5	9, 7	3.2
#1271	188	73	3	3, 7	3.3
#1276	175	92	1	1, 1	2.0
#1412	77	88	2	4, 3	3.5
#1605	125	85	2	6, 5	5.5
#1833	236	109	5	5, 12	3.4

Fig. 5. Successful spermatid orientation, alignment and release at stage VIII and IX of the spermatogenic cycle in rescued adult testes and morphology of isolated spermatozoa from rescued adult mice. (**A**,**B**) Histological sections from the testis and epididymis of 4-month-old rescued mice. Successful spermatid alignment and release at stage VIII (A) and XI (B). (**C-G**) Spermatozoa from wild-type (C), mutant (D-F) and rescued (G) mice were examined after one step Eosin-Nigrosin staining. Defective sperm heads in mutant mice were noted (D-F), with bent or blunted sharp (E), or round or ovoid (F) heads. By contrast, normal sickle-shaped heads with apical hooks were found in rescued males, similar to wild type (G versus C). Magnification: ×60. The arrow points to the thinned mid-piece of the tail. Arabic numerals indicate the step of spermatids shown. P, pachytene spermatocytes; PL/L, pre-leptotene/leptotene spermatocytes.

USA) were used as recipients and there was no apparent immunological rejection of the donor cells (Fig. 7A,C,E). $Rara^{+/+}$; $EGFP^{+}$ germ-cell stem cells were able to repopulate and undergo spermatogenesis readily in $Rara^{+/+}$ (Fig. 7A) and $Rara^{+/-}$ (Fig. 7C,E) germ-cell-depleted testes examined 3.5 months after transplantation (Fig. 7B,D,F). By contrast, no $Rara^{+/+}$; $EGFP^{+}$ germ cells were detected in $Rara^{-/-}$ (Fig. 7G,H) germ-cell-depleted testes 3.5 months after transplantation (Fig. 7H). This suggested that RARα was required in somatic cells to support the niche or proper cellular interaction for spermatogonial development in the seminiferous tubules.

DISCUSSION

We have previously shown that $Rara^{-/-}$ testes exhibit numerous defects in spermatogenesis, and prominent abnormalities in spermiogenesis in particular. Characteristic abnormalities include defects in the orientation of step 8-9 spermatids with regard to the basal aspect of Sertoli cells, randomly oriented spermatids in stage VIII-IX tubules, a failure of spermatid alignment at the lumen in
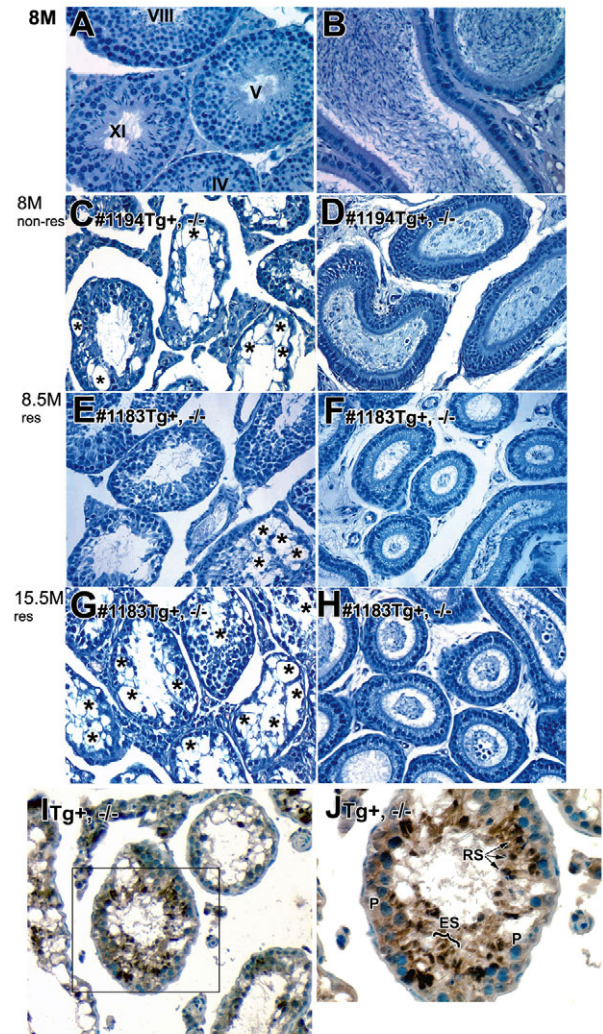
Fig. 6. Sloughing of spermatogenic cell layers in the testes of older rescued mice. (**A**,**B**) Histological sections of testes and epididymis of control male at 8 months. (**C**,**D**) As early as 8.5 months, striking sloughing of spermatogenic cell layers was detected in non-rescued testes (C) and no spermatozoa were found in the corresponding epididymis (D). (**E-H**) Histological sections from testes of rescued $Rara^{-/-}$; $Prm1$-$Rara$/$EGFP$ mice at 8.5 months (E) and 15.5 months (G) are shown with their corresponding epididymides (F and H, respectively). (**I**,**J**) Immunostaining of testes of rescued but now infertile $Rara^{-/-}$; $Prm1$-$Rara$/$EGFP$ mice using GFP antibody. J represents a higher magnification of the insert in I. Asterisks in C, E and G mark the vacuoles in the tubules. Magnification: A-I, ×40; J, ×60. ES, elongated spermatids; non-res, non-rescued mice; P, pachytene spermatocytes; res, rescued mice; RS, round spermatids.

stage VIII tubules, and defects in spermiation (Chung et al., 2004; Chung et al., 2005). In the present study, we extended this analysis to characterize the morphological abnormalities of those spermatozoa that were in the epididymides, suggesting that morphogenetic events were also affected by retinoid signaling. Together, these findings suggested that spermiogenesis is exquisitely sensitive to defects in retinoid signaling.

Using genetically manipulated animal models, we have now shown that restoring RARα expression in haploid spermatids of otherwise $Rara^{-/-}$ mice was able to rescue spermatogenic
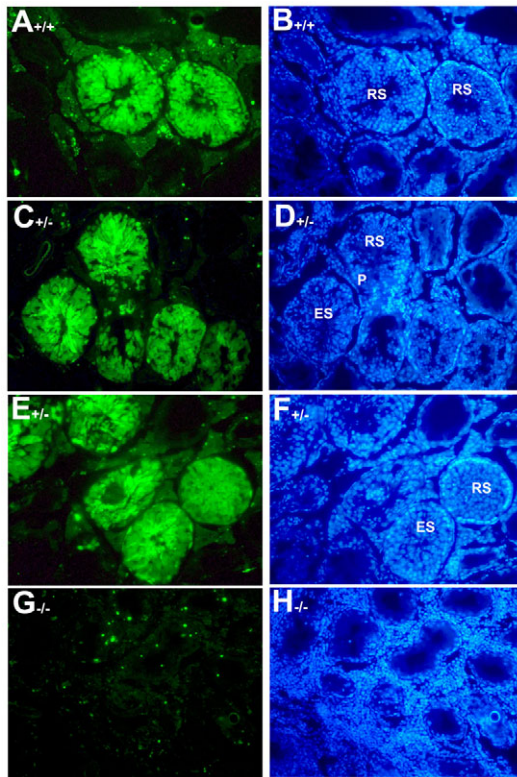
Fig. 7. RARα in the somatic-cell lineage is essential for normal donor germ-cell stem cells to colonize the *Rara*^{−/−} germ-cell-depleted recipient seminiferous tubules. (**A-H**) Green-fluorescing cells were detected in some but not all tubules of *Rara*^{+/+} (A) and *Rara*^{+/−} (C,E) germ-cell-depleted recipient testes, as examined 3.5 months after transplantation, indicating the successful colonization of transplanted cells. The right panel showed the same tubules as the corresponding left panel but counterstained with DAPI (B, D and F, respectively). No green-fluorescing cells were noted in the *Rara*^{−/−} germ-cell-depleted recipient testes examined 3.5 months after transplantation (G), and Sertoli-cell-only tubules were noted after the same tubules were counterstained with DAPI (H). A-H, ×20. ES, elongated spermatids; P, pachytene spermatocytes; RS, round spermatids.

differentiation. In several of these mice, fertility was restored and progeny were produced. To our knowledge, this is the first report demonstrating the requirement of RARα-mediated signaling in a specific developmental stage within the germ-cell lineage, namely haploid spermatids. This further supported the notion that RARα function in the germline is essential in regulating the mechanism for spermatid orientation, alignment and release. It is important to note that this rescue by a transgenic model was not fully penetrant. Such a variable penetrance has been reported in a transgenic haploid germ-cell-specific rescue of mice null for the testicular form of hormone-sensitive lipase (HSL$_{tes}$) (Vallet-Erdtmann et al., 2004). That is, mice with one allele containing the HSL$_{tes}$ transgene(s) were variably infertile and produced small litters. However, two copies of the transgenic allele were able to restore fertility with normal litter sizes. RARα may be involved in the maintenance of some homeostatic processes (Dollé et al., 1990; Ruberte et al., 1991), as the size of the litters from homozygous *Rara*^{−/−} females and heterozygous *Rara*^{+/−} males is smaller (5.50±0.75) relative to wild-type mating on a similar mixed background (11.90±2.16).

The temporal progression of spermatogenesis is rigidly controlled but poorly understood and includes characteristic timing of mitotic and meiotic cell cycles and resulting defined cellular associations (Oakberg, 1956; Russell et al., 1990). Temporary arrest in the progression of spermatogenesis has been demonstrated in VAD rat testes, suggesting that this tight regulation involves retinoid signaling (de Rooij et al., 1994; Ismail et al., 1990; Morales and Griswold, 1987). The abnormal cellular associations in RARα-deficient testes further indicated that retinoid signaling mediated by RARα is essential in this process (Chung et al., 2004). Germ-cell transplantation has been used to demonstrate that the genetic control of the timing of the spermatogenic cycle is intrinsic to the germ cells (Franca et al., 1998). Using this transplantation approach, we demonstrated that although *Rara*^{−/−} germ-cell stem cells can initiate spermatogenesis, the ensuing differentiation results in improper cellular associations, highlighting the idea that RARα function in the germ-cell lineage seemed to be involved in the maintenance of normal cellular association.

We further demonstrated that although *Rara*^{−/−} germ cells transplanted into *Rara*^{+/+} recipient mice progressed to the elongating spermatid stage, their morphology was abnormal. As the somatic cells in these experiments were wild-type for RARα function, this suggested that RARα function in the germline is crucial for certain morphogenetic processes. Concomitantly, using transgenic approaches, we showed that restoring RARα function in haploid spermatids was able to rescue differentiation of haploid germ cells to form normal spermatozoa.

A recent study in which *RAR*α^{−/−} (C56Bl/6) germ cells were transplanted into W/W^v (WBB6F1/J-Kit^W/Kit^{W-v}) testes reported a very low transplantation efficiency (1 out of the 14 recipient mice had donor-derived colonization, and only one region of the tubule from this mouse was colonized) (Doyle et al., 2007). This is surprising in light of our more successful transplantation results. The resulting very low efficiency of colonization in those studies may reflect experimental artifact rather than RARα status of the donor cells for several reasons. First, a suboptimal age of donor cells (6-month-old *Rara*^{−/−} germ cells) was used, which may affect transplantation efficiency. Second, it has been shown by us and others that there is considerable testicular degeneration in RARα-deficient mice by 6 months of age (Lufkin et al., 1993; Chung et al., 2004; Doyle et al., 2007).

This study by Doyle et al. (Doyle et al., 2007) also reported that *RAR*α^{+/+} germ cells from B6/129-TgR(Rosa26)26Sor (Rosa26) mice were able to colonize B6 6-month-old *Rara*^{−/−} testes. These Rosa26 germ cells with mixed background would not be predicted to transplant efficiently into RARα-deficient mice testes on a pure C57BL/6 background. In fact, only small regions of the tubules were positive for donor cells, and no tubules with more advanced donor cells positive for β-galactosidase were reported (Doyle et al., 2007). In addition, it was impossible to evaluate the source of the more advanced spermatogenic cells that were present, as immunohistochemistry using polyclonal anti-RARα peptide antibodies (Santa Cruz, CA, USA) was used in this study instead of X-gal staining to detect repopulated Rosa26 spermatogenic cells at more advanced stages. The specificity of these antibodies is questionable (Vernet et al., 2006b), and further, a non-optimal age of RARα-deficient recipient mice (6 months old) appeared to have been used in this study (Doyle et al., 2007). No busulfan treatment of the recipient mice was mentioned, and the presence of endogenous *Rara*^{−/−} germ cells in RARα-deficient recipient mice may result in a different niche or environment for the donor Rosa26 germ cells. As such, the resulting more advanced stages reported by Doyle et al.

(Doyle et al., 2007) may simply reflect experimental artifact rather than the role of RARα in somatic cells of the recipient testes. Although it was reported that the majority of donor-derived cell types were early meiotic prophase spermatocytes and there were fewer round and elongated spermatids, in fact the authors also raised doubts with regard to their observations (Doyle et al., 2007). In particular, they noted that their results suggesting that RARα only in germ cells (without RARα in the somatic-cell lineage) was not sufficient for meiosis and maturation of spermatids seemed counterintuitive, because the expression of RARα is highest in early primary spermatocytes and elongating spermatids (Akmal et al., 1997; Dufour and Kim, 1999).

By contrast, our germ-cell transplantation studies demonstrated that *Rara*$^{-/-}$ somatic cells failed to support the repopulation and reinitiation of transplanted normal germ cells. Whether this reflects an absolute requirement for RARα in the somatic compartment or, alternatively, reflects a decreasing ability of the somatic compartment to support spermatogenesis with age remains to be determined. However, we favor the latter hypothesis for the following reasons. To use *Rara*$^{-/-}$ mice as recipients for germ-cell transplantation, the mice were treated with busulfan at 4-6 weeks of age, and an additional 4 weeks were needed to obtain tubules devoid of endogenous germ cells for transplantation. After transplantation, another 3-5 months were required for colonies of donor-cell-derived spermatogenesis to be established. As such, these *Rara*$^{-/-}$ recipient mice were now around 6 months of age, an age at which tubules with extensive vacuoles and sloughing of germ cells is seen in *Rara*$^{-/-}$ testis (Chung et al., 2004; Lufkin et al., 1993). Concomitantly, we also observed severe sloughing of germ-cell layers in the testes of rescued *Rara*$^{-/-}$; *Prm1-Rara/EGFP*$^{+}$ mice with time, as also observed in *Rara*$^{Ser-/-}$ mutant tubules (Vernet et al., 2006a). Interestingly, this phenotype is reminiscent of the germ-cell-depleted or Sertoli-cell-only tubules described in the *Rara*$^{-/-}$ and VAD testes (reviewed by Chung and Wolgemuth, 2004). As somatic cells in the above-mentioned mouse models are RARα-deficient, these observations also suggest a possible role for RARα in the somatic-cell lineage in the maintenance of germinal epithelium.

To our knowledge, this is the first report providing direct evidence for the distinctive requirement of RARα-mediated retinoid signaling in germ cells for their normal differentiation. Given the impaired spermiogenesis in *Rara*$^{-/-}$ mice, the defects might result from the downregulation of retinoid-mediated target genes in round spermatids. Our transgenic model that expresses RARα in round and elongating spermatids will provide a useful tool to dissect the retinoid signaling pathways involved in germ-cell differentiation by, for example, genome-wide microarray expression analysis (Chalmel et al., 2007). Such analyses ultimately will expand our understanding of the transcriptional network regulating spermatogenesis and the unique role of RARα in this differentiation.

References

Akmal, K. M., Dufour, J. M. and Kim, K. H. (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.

Akmal, K. M., Dufour, J. M., Vo, M., Higginson, S. and Kim, K. H. (1998). Ligand-dependent regulation of retinoic acid receptor alpha in rat testis: in vivo response to depletion and repletion of vitamin A. *Endocrinology* **139**, 1239-1248.

Bjorndahl, L., Soderlund, I. and Kvist, U. (2003). Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum. Reprod.* **18**, 813-816.

Braun, R. E., Peschon, J. J., Behringer, R. R., Brinster, R. L. and Palmiter, R. D. (1989). Protamine 3′-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. *Genes Dev.* **3**, 793-802.

Brinster, R. L. and Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA* **91**, 11303-11307.

Chalmel, F., Rolland, A. D., Niederhauser-Wiederkehr, C., Chung, S. S., Demougin, P., Gattiker, A., Moore, J., Patard, J. J., Wolgemuth, D. J., Jegou, B. et al. (2007). The conserved transcriptome in human and rodent male gametogenesis. *Proc. Natl. Acad. Sci. USA* **104**, 8346-8351.

Cheng, C. Y. and Mruk, D. D. (2002). Cell junction dynamics in the testis: sertoli-germ cell interactions and male contraceptive development. *Physiol. Rev.* **82**, 825-874.

Chung, S. S. and Wolgemuth, D. J. (2004). Role of retinoid signaling in the regulation of spermatogenesis. *Cytogenet. Genome Res.* **105**, 189-202.

Chung, S. S., Sung, W., Wang, X. and Wolgemuth, D. J. (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.

Chung, S. S., Wang, X. and Wolgemuth, D. J. (2005). Male sterility in mice lacking retinoic acid receptor alpha involves specific abnormalities in spermiogenesis. *Differentiation* **73**, 188-198.

Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J. and Pandolfi, P. P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat. Genet.* **36**, 653-659.

Crusio, W. E. (2004). Flanking gene and genetic background problems in genetically manipulated mice. *Biol. Psychiatry* **56**, 381-385.

de Rooij, D. G., van Pelt, A. M. M., Van de Kant, H. J. G., van der Saag, P. T., Peters, A. H. F. M., Heyting, C. and de Boer, P. (1994). Role of retinoids in spermatogonial proliferation and differentiation and the meiotic prophase. In *Function of Somatic Cells in the Testis* (ed. A. Bartke), pp. 345-361. New York: Springer.

Dollé, P., Ruberte, E., Leroy, P., Morriss-Kay, G. and Chambon, P. (1990). Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**, 1133-1151.

Doyle, T. J., Braun, K. W., McLean, D. J., Wright, R. W., Griswold, M. D. and Kim, K. H. (2007). Potential functions of retinoic acid receptor A in sertoli cells and germ cells during spermatogenesis. *Ann. NY Acad. Sci.* **1120**, 114-130.

Dufour, J. M. and Kim, K. H. (1999). Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. *Biol. Reprod.* **61**, 1300-1308.

Eskild, W. and Hansson, V. (1994). Vitamin A functions in the reproductive organs. In *Vitamin A in Health and Disease* (ed. R. Blomhoff), pp. 531-559. New York: Dekker.

Fajardo, M. A., Haugen, H. S., Clegg, C. H. and Braun, R. E. (1997). Separate elements in the 3′ untranslated region of the mouse protamine 1 mRNA regulate translational repression and activation during murine spermatogenesis. *Dev. Biol.* **191**, 42-52.

Franca, L. R., Ogawa, T., Avarbock, M. R., Brinster, R. L. and Russell, L. D. (1998). Germ cell genotype controls cell cycle during spermatogenesis in the rat. *Biol. Reprod.* **59**, 1371-1377.

Griswold, M. D., Bishop, P. D., Kim, K. H., Ping, R., Siiteri, J. E. and Morales, C. (1989). Function of vitamin A in normal and synchronized seminiferous tubules. *Ann. NY Acad. Sci.* **564**, 154-172.

Howell, J. M., Thompson, J. N. and Pitt, G. A. J. (1963). Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid, I. The male rat. *J. Reprod. Fertil.* **5**, 159-167.

Ismail, N., Morales, C. and Clermont, Y. (1990). Role of spermatogonia in the stage-synchronization of the seminiferous epithelium in vitamin-A-deficient rats. *Am. J. Anat.* **188**, 57-63.

Jeffs, B., Ito, M., Yu, R. N., Martinson, F. A., Wang, Z. J., Doglio, L. T. and Jameson, J. L. (2001). Sertoli cell-specific rescue of fertility, but not testicular pathology, in Dax1 (Ahch)-deficient male mice. *Endocrinology* **142**, 2481-2488.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Ogura, A., Toyokuni, S., Honjo, T. and Shinohara, T. (2003). Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. *Biol. Reprod.* **68**, 167-173.

Liao, C., Wang, X. Y., Wei, H. Q., Li, S. Q., Merghoub, T., Pandolfi, P. P. and Wolgemuth, D. J. (2001). Altered myelopoiesis and the development of acute myeloid leukemia in transgenic mice overexpressing cyclin A1. *Proc. Natl. Acad. Sci. USA* **98**, 6853-6858.

Livera, G., Rouiller-Fabre, V., Pairault, C., Levacher, C. and Habert, R. (2002). Regulation and perturbation of testicular functions by vitamin A. *Reproduction* **124**, 173-180.

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

Morales, C. and Griswold, M. D. (1987). Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology* **121**, 432-434.

Oakberg, E. F. (1956). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391-409.

Ogawa, T., Arechaga, J. M., Avarbock, M. R. and Brinster, R. L. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. *Int. J. Dev. Biol.* **41**, 111-122.

O'Gorman, S., Dagenais, N. A., Qian, M. and Marchuk, Y. (1997). Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94**, 14602-14607.

Ohta, H., Yomogida, K., Yamada, S., Okabe, M. and Nishimune, Y. (2000). Real-time observation of transplanted 'green germ cells': proliferation and differentiation of stem cells. *Dev. Growth Differ.* **42**, 105-112.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Ruberte, E., Dolle, P., Chambon, P. and Morriss-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111**, 45-60.

Russell, L. D., Ettlin, R. A., SinhaHikim, A. P. and Clegg, E. D. (1990). Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press.

Shang, E., Nickerson, H. D., Wen, D., Wang, X. and Wolgemuth, D. J. (2007). The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. *Development* **134**, 3507-3515.

Vallet-Erdtmann, V., Tavernier, G., Contreras, J. A., Mairal, A., Rieu, C., Touzalin, A. M., Holm, C., Jegou, B. and Langin, D. (2004). The testicular form of hormone-sensitive lipase HSLtes confers rescue of male infertility in HSL-deficient mice. *J. Biol. Chem.* **279**, 42875-42880.

van Pelt, A. M., van den Brink, C. E., de Rooij, D. G. and van der Saag, P. T. (1992). Changes in retinoic acid receptor messenger ribonucleic acid levels in the vitamin A-deficient rat testis after administration of retinoids. *Endocrinology* **131**, 344-350.

Vernet, N., Dennefeld, C., Guillou, F., Chambon, P., Ghyselinck, N. B. and Mark, M. (2006a). Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *EMBO J.* **25**, 5816-5825.

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

Wolbach, S. B. and Howe, P. R. (1925). Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* **42**, 753-777.

Wolfer, D. P., Crusio, W. E. and Lipp, H. P. (2002). Knockout mice: simple solutions to the problems of genetic background and flanking genes. *Trends Neurosci.* **25**, 336-340.

Wolgemuth, D. J. and Chung, S. S. (2007). Retinoid signaling during spermatogenesis as revealed by genetic and metabolic manipulations of retinoic acid receptor alpha. *Soc. Reprod. Fertil. Suppl.* **63**, 11-23.

Wolgemuth, D. J., Gizang-Ginsberg, E., Engelmyer, E., Gavin, B. J. and Ponzetto, C. (1985). Separation of mouse testis cells on a Celsep (TM) apparatus and their usefulness as a source of high molecular weight DNA or RNA. *Gamete Res.* **12**, 1-10.

Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989). Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature* **339**, 714-717.