Abnormal spermatogenesis in RXRβ mutant mice

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We have generated mouse lines in which the RXRβ gene was disrupted by homologous recombination. Approximately 50% of the RXRβ homozygous mutants died before or at birth, but those that survived appeared normal except that the males were sterile, owing to oligo-astheno-teratozoospermia. Failure of spermatid release occurred within the germinal epithelium, and the epididymis contained very few spermatozoa that, in addition, exhibited abnormal acrosomes and tails. There was a progressive accumulation of lipids within the mutant Sertoli cells, which were histochemically characterized as unsaturated triglycerides. In old mutant males, progressive degeneration of the germinal epithelium occurred, ending with the formation of acellular lipid-filled tubules. The selective expression of RXRβ in Sertoli cells, together with the timing of appearence of the histological abnormalities, suggests that the primary defect resulting from the mutation resides in these cells.

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The three retinoid X receptors, RXRα, RXRβ, and RXRγ, are members of the vertebrate nuclear receptor superfamily (Leid et al. 1992a,b; Mangelsdorf et al. 1992, 1994; Chambon 1994; Giguère 1994; Glass 1994; Kastner et al. 1994b). In the mouse, RXRα and RXRβ transcripts appear to be widely expressed in the embryo and in adult tissues, whereas the distribution of RXRγ transcripts is more restricted (Mangelsdorf et al. 1992; Liu and Linney 1993; Dollé et al. 1994; Nagata et al. 1994). In vitro DNA-binding studies and studies in transfected cells cultured in vitro have provided evidence indicating that RXRs could be involved in several signaling pathways. First, as 9-*cis* retinoic acid (RA) (9C-RA)-dependent transcriptional regulators, RXR homodimers may transduce some of the effects of the active retinoid derivatives of vitamin A. Second, RXRs may also play a role in the retinoid signaling pathway, as heterodimeric partners for the retinoic acid receptors (RARα, RARβ, and RARγ) that act as all-*trans* RA (T-RA)- or 9C-RA-dependent transcriptional regulators. Third, RXRs may be involved as heterodimeric partners in additional signaling pathways mediated by other nuclear receptors, which include the thyroid hormone receptors (TRs), the vitamin D3 receptor (VDR), and the peroxisome proliferator-activated receptors (PPARs) (for review, see Mangelsdorf et al. 1992; Liu and Linney 1993; Dollé et al. 1994; Nagata et al. 1994; Desvergne and Wahli 1995). Finally, RXRs may also be the heterodimeric partners of a number of orphan nuclear receptors, including members of the FXR/RLD-1/LXR/UR family (Apfel et al. 1994; Song et al. 1994; Forman et al. 1995a; Seol et al. 1995; Teboul et al. 1995; Willy et al. 1995; note that UR has also been named OR-1 or RIP15), members of the NGFI-B/NURR-1 family (Forman et al. 1995b; Perlmann and Jansson 1995), and the orphan receptor MB67 (Baes et al. 1994).

Whether RXRs engaged in heterodimeric associations act as 9C-RA-dependent transcriptional regulators to synergistically control initiation of transcription or are transcriptionally silent, simply allowing their partners to bind efficiently to the response elements of their cognate target genes, is largely unknown. Several studies performed in vitro and in transfected cultured cells have suggested that the 9C-RA-dependent transcriptional activity of the RXR partner is dependent on both the nature and the ligand occupancy of the heterodimeric partner, as well as on the nature of the bound response element (Durand et al. 1994; Kurokawa et al. 1994; Forman et al. 1995b; Perlmann and Jansson 1995; Willy et al. 1995). Interestingly, Roy et al. (1995) have shown recently that, in P19 and F9 embryonal carcinoma cells, RAR- and RXR-specific ligands have synergistic effects to activate

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endogenous RA target genes and to induce differentiation, which indicates that, at least in some cases of RXR/RAR partnerships, both receptors can be implicated in transcriptional activation of target genes.

In contrast to the wealth of information related to the functions of RXRs in vitro, very little is known to date concerning the actual physiological role of RXRs in vivo. Notably, it is unknown whether, as a receptor for 9C-RA, RXR transduces some of the multiple effects of retinoids. In this respect, it is noteworthy that most of the abnormalities found in vitamin A-deficient fetuses or adult mice are reproduced in mice bearing mutations in one or several RARs, which demonstrate that RARs are involved in the physiological transduction of the RA signal (Lohnes et al. 1993, 1994; Lufkin et al. 1993; Mendelsohn et al. 1994). The possible function of RXRα has been investigated by examining homozygous null mice generated by targeted disruption of the RXRα gene in embryonic stem (ES) cells (Kastner et al. 1994a; Sucov et al. 1994). These mutants exhibit fetal heart and eye defects similar to those occurring in the fetal vitamin A-deficiency (VAD) syndrome, thus suggesting that RXRα mediates some of the effects of vitamin A. Moreover, these studies have revealed a strong synergy between mutations in RAR and RXR genes for the generation of abnormalities that were either less severe in single RXR or RAR mutants or not present at all. It was also shown that some specific RXR/RAR pairs are much more efficient than others at generating some of the abnormalities seen in the fetal VAD syndrome (Kastner et al. 1994a; P. Kastner, M. Mark, and P. Chambon, unpubl.). Taken together, these data strongly suggest that RAR/RXR heterodimers are the major functional units responsible for transducing the retinoid signal during development.

We now report the generation of RXRβ null mutant mice. Approximately 50% of these mutants die in utero or very shortly after birth for unknown reasons. Surprisingly, the remaining null mutants are externally indistinguishable from their wild-type or heterozygous littermates. However, RXRβ$^{-/-}$ males are sterile, owing to abnormal germ cell maturation, leading to oligo-astheno-teratozoospermia. These sperm abnormalities most probably reflect an indispensable function of RXRβ in the Sertoli cells, which in prepubertal mutant males start to accumulate lipid droplets. The possible involvement of PPARs in the generation of the testis abnormalities seen in RXRβ null mutants is discussed.

Results

RXRβ mutant mice

The RXRβ gene was disrupted by homologous recombination in ES cells with a replacement-type vector in which the genomic sequences encoding the DNA-binding domain (3′ region of exons 3 and 4) were replaced with a PGK–NEO(A$^+$) cassette (Fig. 1a,b). Chimeric males derived from two mutant ES clones (HA67 and HA9) transmitted the mutation, thus generating two lines of mutant mice. For both lines, crosses between heterozygotes (RXRβ$^{+/-}$) yielded viable homozygote mutants (RXRβ$^{-/-}$), which were externally indistinguishable from their wild-type and heterozygote littermates. Homozygote females were fertile, but males were sterile (see below).

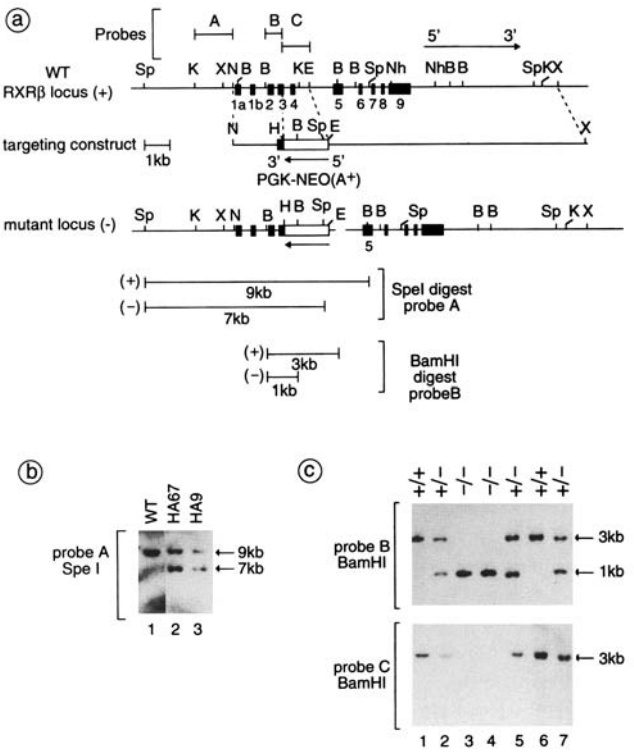

Figure 1. The RXRβ mutation. (*a*) Targeting the RXRβ gene. A map of the wild-type RXRβ gene is shown at the *top*. (■) Exons (numbering as in Nagata et al. 1994). Probe A is a *Not*I–*Kpn*I fragment; probe B is a 500-bp *Bam*HI–*Hin*dIII fragment immediately upstream of the deleted region; probe C is the *Hin*dIII–*Eco*RI fragment (corresponding to the deleted region). (Sp) *Spe*I; (K) *Kpn*I; (N) *Not*I; (B) *Bam*HI; (E) *Eco*RI; (Nh) *Nhe*I; (X) *Xho*I; (H) *Hin*dIII. (*b*) Targeted ES cells. Southern blot analysis of *Spe*I-restricted DNA from wild type (WT) and the HA67 and HA9 ES cell clones, analyzed with the 5′ probe A. (*c*) Southern blot analysis of mutant mice. (*Top*) The analysis of a litter using *Bam*HI-restricted DNA and probe B. (*Bottom*) Corresponds to an identical blot hybridized with probe C.

As expected, a probe spanning the genomic region encoding the RXRβ DNA-binding domain did not detect any hybridizing fragment in homozygotes (probe C in Fig. 1c). Therefore, even though RXRβ exons 1, 2, and 5–9 are still present in the genome of homozygotes, a functional DNA-binding RXRβ protein cannot be produced in the mutants. RT–PCR analysis of total RNA isolated from several tissues showed that transcripts corresponding to exon 2, as well as transcripts containing exons 5–9, were present at a low level in RXRβ$^{-/-}$ mice when compared with wild type (<10%; data not shown). We cannot therefore exclude that some RXRβ peptides

could be present at low levels in mutants. In any event, it is unlikely that the abnormalities observed might result from a dominant effect of such putative peptides, because heterozygous animals never displayed any defect. Thus, the present disruption of the RXRβ gene most probably corresponds to a null mutation.

Embryonic and perinatal death of a fraction of RXRβ$^{-/-}$ mutants

When genotyped at 2–3 weeks of age, the numbers of RXRβ$^{-/-}$ mutants derived from intercrosses between RXRβ$^{+/-}$ males and either RXRβ$^{+/-}$ or RXRβ$^{-/-}$ females were significantly lower ($P < 0.005$) than expected from a Mendelian ratio (Table 1). A significant deficit was also observed among 1 day-old newborns and 18.5 dpc (days post–coitum) cesarean-delivered fetuses (Table 1), suggesting that some of the missing RXRβ$^{-/-}$ mutants died in utero or shortly after birth.

It appears therefore that RXRβ is important for embryonic development in a fraction of embryos. It is presently unclear when this embryonic death occurs and whether the incomplete penetrance of that phenotype results from heterogeneity in the genetic background of the mutants. That the deficit in RXRβ$^{-/-}$ animals is more pronounced in newborns than in caesarean-delivered 18.5 dpc fetuses (Table 1) suggests that death at or shortly after birth also accounts for some of the missing RXRβ$^{-/-}$ mutants. It is noteworthy, however, that the cesarean-delivered mutants had a normal appearence, breathed normally, and could survive for at least 12 hr when left in isolation. Thus, some of the homozygote mutants may be eliminated through selective maternal cannibalism, similarly to what had been reported previously in the case of RARγ mutants (Lohnes et al. 1993). Even though a few RXRβ$^{-/-}$ mice died during the first weeks after birth, the majority of RXRβ$^{-/-}$ mutants that survived through the gestational and perinatal periods had a viability comparable with that of wild-type or heterozygous littermates.

Table 1. *Viability of RXRβ mutants*

	RXRβ mutant intercrosses				
	♀ +/− × ♂ +/−			♀ −/− × ♂ +/−	
	+/+	+/−	−/−	+/−	−/−
~3-week-old	132	288	73	128	38
	(1)	(2.2)	(0.55)	(1)	(0.3)
1-day-old	15	33	5	70	29
	(1)	(2.2)	(0.3)	(1)	(0.4)
18.5 dpc fetuses	76	129	56	66	39
	(1)	(1.7)	(0.7)	(1)	(0.6)
Expected Mendelian ratio	1	2	1	1	1

The numbers and genotypes of living mice (3 weeks old or 1 day old) or fetuses (delivered by cesarean section at gestational day 18.5) are given. The values (in parentheses) indicate the ratio with respect to the number of wild type (heterozygote intercrosses) or heterozygotes (homozygote/heterozygote crosses).

Oligo-astheno-teratozoospermia and abnormal spermiogenesis in RXRβ$^{-/-}$ males

Seven RXRβ$^{-/-}$ males were tested for fertility over a 6-month period. None sired any offspring, even though each of them produced many vaginal plugs. The testes of all RXRβ$^{-/-}$ males were normally descended in the scrotum; the genital ducts (i.e., epididymis, vas deferens) and the accessory glands (i.e., seminal vesicle, prostate and bulbourethral glands) were macroscopically and histologically normal (E in Fig. 2b; results not shown).

In contrast to the epididymides of adult wild-type mice, which on histological sections were packed with sperm (H in Fig. 2a), those of adult RXRβ$^{-/-}$ mutants (2–10 months of age) contained only sparse, elongated or sickle-shaped nuclear profiles characteristic of differentiated spermatozoa (H in Fig. 2b) along with unidentified cellular debris. Moreover, whereas almost all spermatozoa removed from wild-type caudal epididymis became highly mobile when suspended in phosphate-buffered saline (see Bishop 1961), ~95% of the RXRβ$^{-/-}$ caudal epididymal spermatozoa remained immobile under the same conditions. A majority of the RXRβ$^{-/-}$ spermatozoa also exhibited a coiling of the tail (Fig 2c, arrowheads), an abnormality that was rarely seen in wild-type counterparts. It is noteworthy that this coiling was not seen in testicular spermatids and therefore presumably occurred in the epididymis.

Additional abnormalities were revealed by electron-microscopic examination of RXRβ$^{-/-}$ epididymal spermatozoa. Many acrosomes (~30%) appeared indented (large arrow in Fig. 2d,f,g) and/or partially detached from the nuclear envelope (cf. A, Fig. 2e with f and g). Some spermatozoa lacked an acrosome (not shown). Indented acrosomes were also seen in few wild-type spermatozoa (~7%), but this indentation was never as marked as in mutants. Thus, the high frequency of such defects in mutant spermatozoa strongly suggests that the attachment of the acrosomal membrane to the nucleus (N) is impaired in RXRβ$^{-/-}$ mutants. In addition to these acrosomal defects, local failure of the mitochondria to arrange helicoidally around the axoneme in the middle piece of the mutant spermatozoa tail was observed in ~1–2% of the spermatozoa (open arrow and inset in Fig. 2d). This defect was never seen in wild-type epididymal spermatozoa. Interestingly, these abnormal acrosomes and mitochondrial sheaths were also found in step 16 spermatids within the testis (not shown) and therefore correspond to defects in the process of spermiogenesis (i.e., the maturation of spermatids to spermatozoa within the germinal epithelium).

Taken together, these data indicate that oligo-astheno-teratozoospermia (i.e., severe reduction in the number, reduced mobility, and high percentage of abnormalities of the spermatozoa in the semen) is responsible for the sterility of RXRβ$^{-/-}$ males.

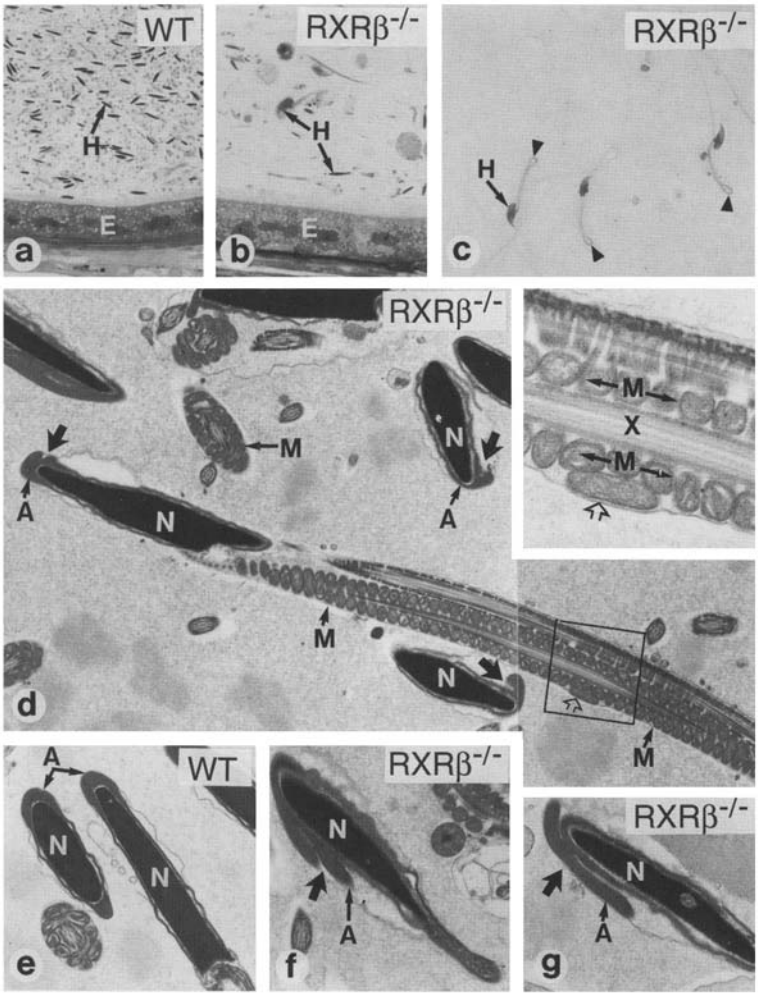

Figure 2. Comparison of spermatozoa from the caudal epididymis in wild-type (WT) and RXRβ$^{-/-}$ mutants, as indicated. (*a,b*) Semithin histological sections, illustrating the paucity of mutant spermatozoa. *c* corresponds to a smear, showing three spermatozoa displaying coiling of their tails. (*d–g*) Thin sections illustrating malformations of the acrosome and mitochondrial sheats in mutant spermatozoa; The *inset* corresponds to a high magnification of the box in *d*. (A) Acrosomes; (E) epididymal epithelium; (H) head of spermatozoa; (M) mitochondrial sheat; (N) nuclei of spermatozoa; (X) axoneme. The arrowheads, the large arrows, and the open arrows point to the coiled tails and the defects of the acrosomes and mitochondrial sheats, respectively. Magnification, 430× (*a–c*), 5000× (*d–g*), and 12,500× (*inset*).

Defective spermiation in the mutant seminiferous tubules

The criteria originally described by Oakberg (1956) and revised by Russell et al. (1990) were used to identify the germ cell types and define the stage of the seminiferous epithelium cycle. In brief, germ cell types were identified primarily by the size and shape of their nuclei, the distribution of chromatin in the nucleus, and the position of the nucleus within the cell. Spermatids were also classified according to the size and shape of the acrosomic system. The latter is strongly periodic acid Schiff (PAS)-positive on sections from Bouin-fixed, paraffin-embedded tissue (A in Fig. 3e,f,i) and is deeply stained by toluidine blue on semithin sections from epon-embedded material (e.g., A in Fig. 3c,d). Round spermatid maturation is divided into eight steps (1–8) reflecting the degree of acrosome development. Elongated spermatid maturation is also divided into eight steps (9–16) on the basis of the shape of the nucleus and acrosome and the extent of head elongation. The stage of a given seminiferous tubule is defined by a specific association of germ cell types. In normal mice, there are 12 stages designated I to XII, each corresponding to one of the first 12 steps of spermiogenesis. At late stage VII, all the nuclei of the elongated spermatids align at the luminal side of the seminiferous epithelium (Fig. 3c), and the spermatozoa are released into the lumen of the seminiferous tubules at early stage VIII. Late stages of the cycle (i.e., IX–XII; see Fig. 3e) only contain one generation of spermatids (instead of two generations at stages I to early stage VIII).

The diameter of the seminiferous tubules of the six 2-month-old and the six 5- to 6-month-old RXRβ$^{-/-}$ males that were analyzed was normal (Fig. 3, cf. T in a and b). The 12 stages of the cycle of the seminiferous epithelium were readily identifiable (Fig. 3c–f; data not shown), and the proportion of the various germ cell types was apparently normal. However, some of the late spermatids failed to align at the luminal side of tubules at late stage VII (unlabeled arrow in Fig. 3d). In addition, almost all stage IX tubules as well as some stage X tubules contained two generations of elongated spermatids, namely, a normal (step 9 or step 10) and a retained generation (step 16; arrows in Fig. 3f). In stage IX tubules, the heads of the retained spermatids were scattered throughout the seminiferous tubule epithelium, being most abundant at the periphery of the tubules, and were often lying close to the basement membrane (arrows in

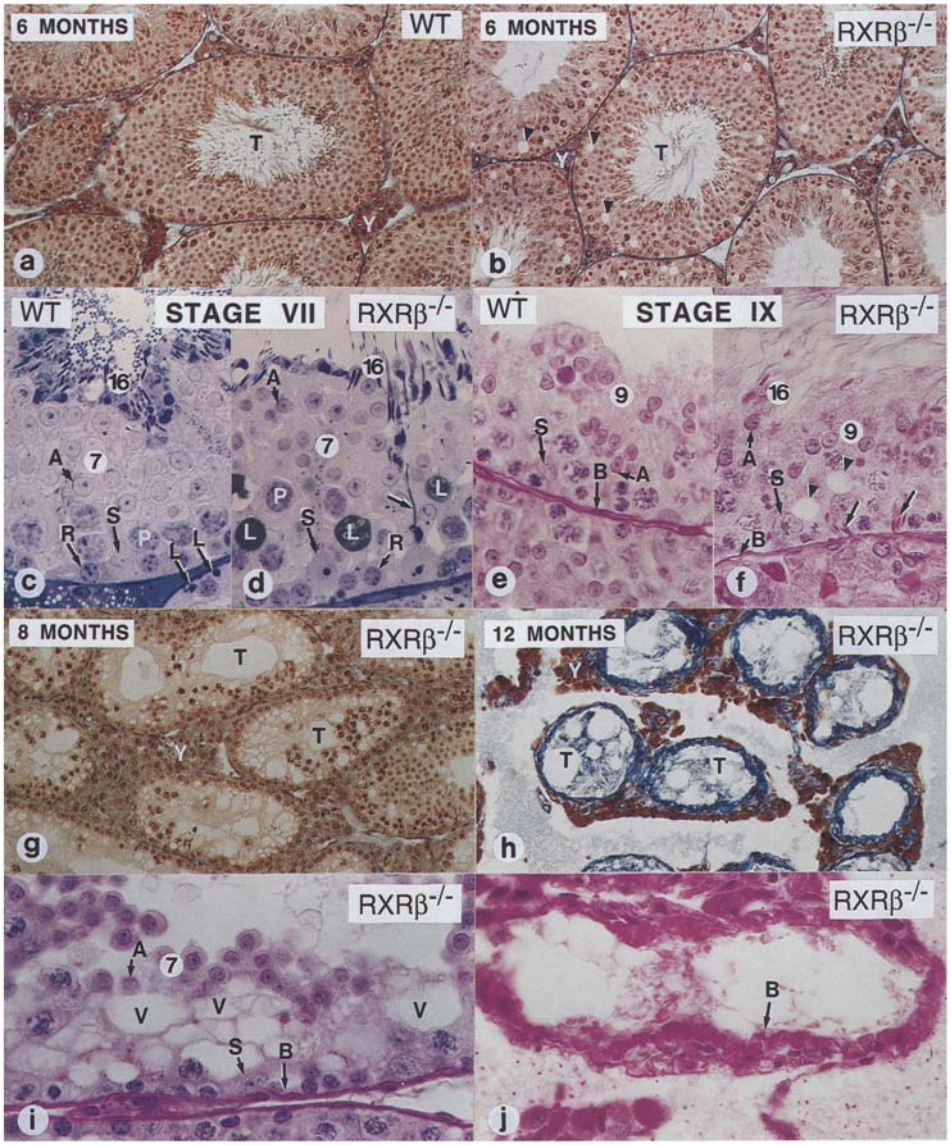

Figure 3. Failure of spermatid release and progressive testicular degeneration in RXRβ$^{-/-}$ males. Sections through the testes of wild-type (WT; *a,c,e*) and RXRβ$^{-/-}$ males (*b,d,f,g–j*) at 6 months (*a–f*), 8 months (*g,i*), and 12 months (*h,j*). (A) Acrosomes; (B) basement membrane; (L) lipid droplets; (P) pachytene spermatocytes; (R) preleptotene spermatocytes; (S) Sertoli cell nucleus; (T) seminiferous tubules; (V) vacuoles of degeneration; (Y) Leydig cells. The numbers in the white dots refer to the degree of maturation (steps) of the spermatids. (Arrowheads in *b,f*) Lipid-containing vacuoles; (unlabeled arrows) retained step 16 spermatids. Note that the focal hyperplasia of the Leydig cells (Y) in *g* and the edema of the intertubular space in *h* represent classical secondary alterations to seminiferous tubule atrophy. Hematoxylin–trichrome (*a,b,g,h*), alcian blue (*c,d*), and PAS–hematoxylin (*e,f,i,j*). Magnification, *a,b,g,* and *h* (111×); *c–f,i,* and *j* (370×).

Fig. 3f). At the electron-microscopic level, these remnants of elongated spermatids were located within the cytoplasm of Sertoli cells (large arrow in Fig. 4). In addition, these phagocytosed spermatids reacted strongly for the detection of fragmented DNA by the TUNEL method (Gavrieli et al. 1992; data not shown). Therefore, defective spermiation (i.e., spermatid release), accompanied by phagocytosis of the retained late spermatids by the Sertoli cells, occurs in RXRβ$^{-/-}$ testes. This defect is likely to account for the near absence of spermatozoa in the epididymides of the RXRβ$^{-/-}$ mice.

Lipid accumulation in mutant Sertoli cells

Paraffin sections of all adult (i.e., 2 months old and older) RXRβ$^{-/-}$ testes revealed also the presence of large, rounded, clear "vacuoles" located at the periphery of every seminiferous tubule (arrowheads in Fig. 3, cf. b and f

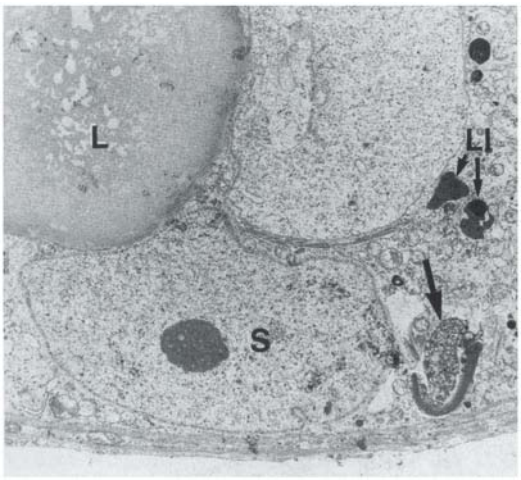

Figure 4. Basal portion of a 6-month-old RXRβ$^{-/-}$ mutant Sertoli cell in a stage IX seminiferous tubule. (L) Lipid droplet; (LI) lysosomes; (S) Sertoli cell nucleus; (arrow) phagocytized elongated spermatid. Magnification, 5400×.

with a and e). These vacuoles were strongly osmiophilic on semithin sections from resin-embedded material (L in Fig. 3d and Fig. 6d,f,g, below), suggesting that they contained lipids. In unfixed frozen sections, they reacted with osmium tetroxide (Fig. 5, cf. T in c and d), thus demonstrating the unsaturated character of the lipids (see Gabe 1968). The lipid dye oil red O, which stains mainly triglycerides and sterols, also stained strongly the tubules of RXRβ$^{-/-}$ mutants (Fig. 5, cf. T in a and b), whereas the Schultz's test for sterols gave a negative response (not shown), suggesting therefore that the droplets contain triglycerides. These droplets were also devoid of phospholipids, because they failed to react with MC22-33F, a monoclonal antibody directed against phosphatidylcholine [Fig 5e; note that the lipids present in granulosa cells, the female equivalent of the Sertoli cells, react strongly with this antibody (G in Fig. 5f)]. Together, these histochemical and immunohistochemical properties of the vacuoles indicate that they essentially correspond to droplets of unsaturated triglycerides. Acid phosphatase positive granules (probably lysosomal

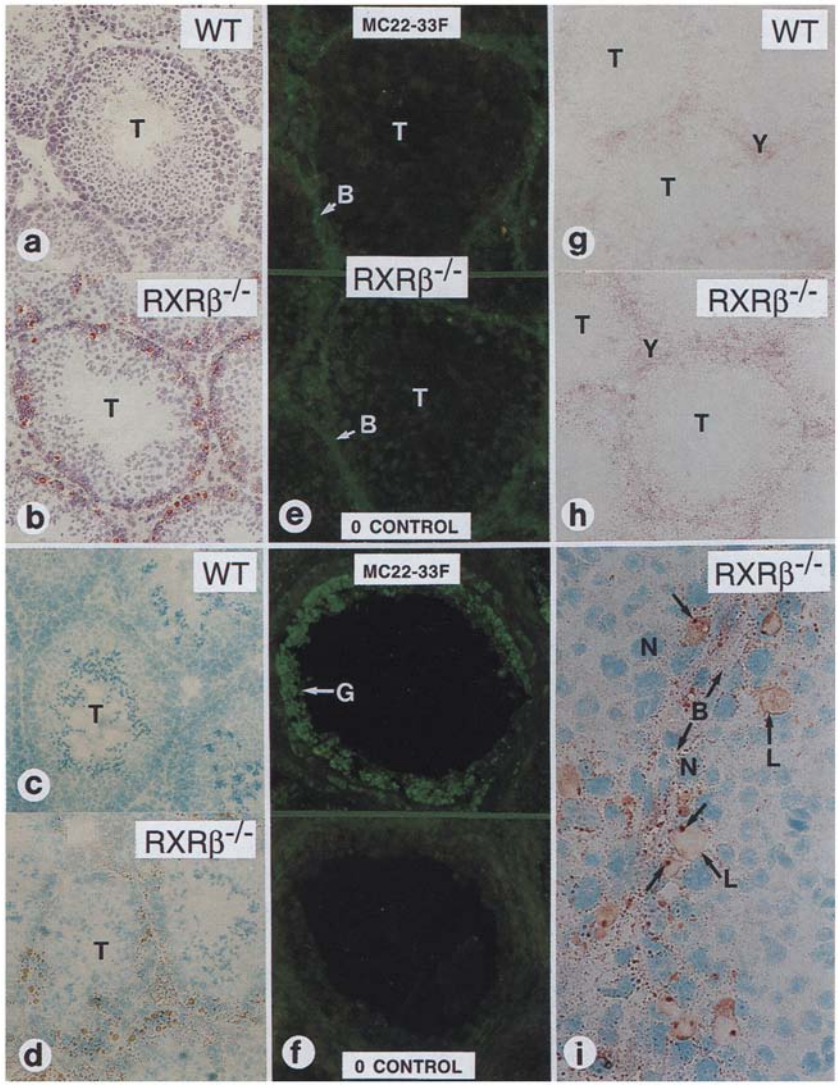

Figure 5. Lipids and lysosomal structures in RXRβ$^{-/-}$ mutant testis. (*a–e, g–i*) Frozen sections through the testes of 6-month-old wild-type (WT; *a,c,g*) and RXRβ$^{-/-}$ (*b,d,e,h,i*) males. (*a,b*) Staining with oil red O. (*c,d*) Staining with osmium tetroxide. (*e*) Immunofluorescence staining of a RXRβ$^{-/-}$ seminiferous tubule with the anti-phospholipid antibody (MC22-33F; *top*) or nonimmune rat IgM (0 control; *bottom*), on consecutive sections. (*f*) Immunostaining of an antral follicle with MC22-33F (*top*) or nonimmune rat IgM (0 control; *bottom*) on consecutive sections. (*g–i*) Detection of acid phosphatase activity (after staining with oil red O in *i*). (B) Level of the basement membrane of the seminiferous tubule; (G) granulosa cells; (L) lipid droplets in Sertoli cells; (N) nuclei; (T) seminiferous tubules; (Y) Leydig cells. Arrows point to lysosomes in *i*. Magnification, 62× (*a–d,g,h*), 96× (*e,f*), and 412× (*i*).

in nature) were consistently associated with the lipid droplets (arrows in Fig. 5i; cf. T in Fig. 5, g and h). It is noteworthy that in wild-type testis both the triglycerides and the acid phosphatase activity were almost exclusively confined to the Leydig cells (Y in Fig. 5g; data not shown). At the electron-microscopic level, these RXRβ$^{-/-}$ tubular triglyceride droplets were strictly confined to the cytoplasm of the Sertoli cells and were not limited by a membrane (L in Fig. 4).

To investigate the evolution of the lipid droplets with age, we examined semithin sections of osmium-fixed testis from 29-day-old animals as well as from 2-, 6-, 8-, and 12-month-old animals (Fig. 6a–h). Lipid droplets were scarce and small in wild-type testes at all ages (see, e.g., L in Fig. 3c). In RXRβ$^{-/-}$ testes, abundant small lipid droplets were already detected in all 29-day-old mutant males analyzed (four animals; Fig. 6, cf. T in a and b). Note that these animals had not yet completed their first round of spermatogenesis (Russel et al. 1990). The size of these lipid droplets increased gradually with the age of the mutant (L in Fig. 6d, f, g), and in 6-month-old males, some of these droplets were already larger than the Sertoli cell nuclei (L in Figs. 3d, 4, and 6f). In old animals, many tubules were devoid of Sertoli cells but filled with lipids (L in Fig. 6h). It appears therefore that the ultimate death of the Sertoli cells is preceded by a progressive increase in lipid content with age.

It has been suggested that the enzyme "hormone-sensitive lipase" (HSL) may be important for controlling lipid metabolism in Sertoli cells, because it is inactivated in cryptorchid testes, in which lipids accumulate within the Sertoli cells (Stenson-Holst et al. 1994). However, Northern blot analysis of HSL transcript levels in 2 month-old males did not reveal any difference between wild-type and RXRβ$^{-/-}$ adult testes (data not shown).

Degeneration of the seminiferous epithelium in old RXRβ mutant mice

Histological sections through both testes of all 8- to 10-month-old RXRβ$^{-/-}$ mice (five males) showed seminiferous tubules with abnormalities similar to those seen in

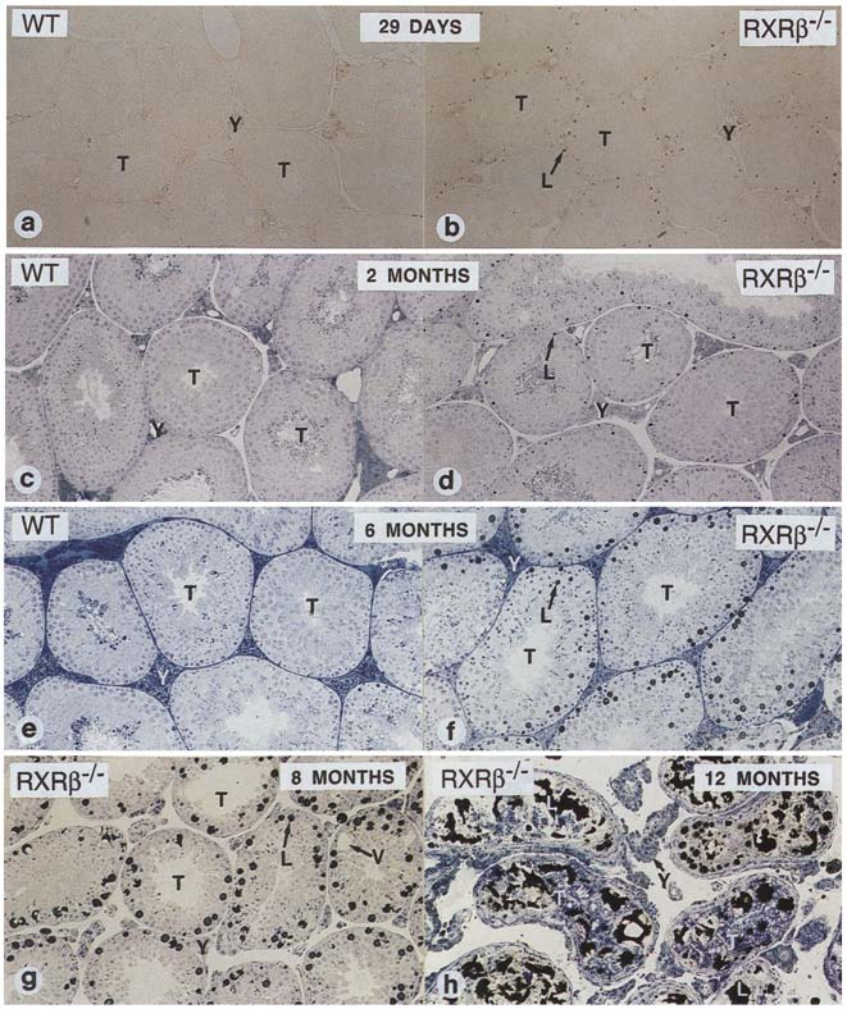

Figure 6. Lipid accumulation in RXRβ$^{-/-}$ mutant testis: Early appearance, prior to the completion of spermatogenesis (29 days), and lipid droplet size increase with the age of the animal, as indicated. Photomicrographs of semithin sections from osmium-fixed testes without (*a,b*) or with counterstaining (*c–h*) with alcian blue. Lipids (L) appear as brown or black dots or spots in the seminiferous tubules (T). (Y) Leydig cells; (V) vacuoles of degeneration. Magnification 66×.

5- to 6-month-old males and, in addition, tubules with reduced diameters that exhibited different degrees of germ cell loss (T in Fig. 3g). These ranged from tubules with normal populations of mitotic (i.e., spermatogonia) and meiotic (e.g., zygotene or pachytene spermatocytes) germ cells, but lacking spermatids, to tubules containing Sertoli cells only. Germ cell loss appeared to result mainly from the sloughing off of apparently healthy immature germ cells (i.e., pachytene spermatocytes and/or round spermatids) into the lumen of the tubules (e.g., step 7 spermatids in Fig. 3i) and was apparently preceded by the appearence of large irregular pockets of empty space in the seminiferous epithelium (V in Fig. 3i). This second type of vacuole, which does not contain lipids (V in Fig. 6g) constitutes in rodent testes an unspecific, early degenerative event (for review, see Yuan and McEntee 1987).

In all testes of 11- to 12-month-old RXRβ$^{-/-}$ males, up to half of the seminiferous tubules were replaced by tubular ghosts consisting of a thickened and convoluted basement membrane, which were filled with lipids (Figs. 3h,j and 6h).

RXRα and RXRβ transcripts' localization and immunolocalization of RXRβ in the seminiferous epithelium

The localization of RXRβ transcripts in the seminiferous epithelium of wild-type males was analyzed by in situ hybridization. RXRβ transcripts were present in every seminiferous tubule (T in Fig. 7b); the strongest signals were detected in the basal portion of the epithelium (where most of the Sertoli cell cytoplasm is located), whereas weaker signals were observed in the more luminal portions (Fig. 7d). Interestingly, the silver grains were often radially aligned in this latter part of the epithelium, which is consistent with a localization in the cytoplasmic processes of Sertoli cells (Fig. 7d; data not shown). Immunoperoxidase staining of wild-type seminiferous tubules with an antibody directed against RXRβ showed a positive signal only in the cell nuclei that on the basis of their localization at the periphery of the seminiferous tubules and their triangular shapes were identified as those of the Sertoli cells. Germ cells [e.g., pachytene spermatocytes (P), round spermatids (RS), and elongated spermatids (ES)] were not stained (Fig. 7f). As expected, no staining was detected in the RXRβ$^{-/-}$ seminiferous epithelium (Fig. 7g). These data indicate that RXRβ testicular expression is restricted to Sertoli cells and does not depend on the stage of the seminiferous tubule.

RXRα transcripts were also present in the testis; however, their localization was apparently restricted to some round spermatids in a subset of tubules (Fig. 7c,e). Therefore, within the seminiferous epithelium, the distributions of RXRα and RXRβ transcripts do not appear to overlap. In contrast, RXRγ transcripts could not be detected in the testis (data not shown).

Discussion

Abnormal spermatogenesis and degeneration of the seminiferous epithelium

We demonstrate here that RXRβ is essential for spermatogenesis in the mouse: RXRβ null mutant males exhibit abnormalities in spermiogenesis (i.e., spermatid maturation) and spermiation (i.e., spermatid release). Interestingly, mutant Sertoli cells exhibit concomitantly an early and a progressive cytoplasmic accumulation of unsaturated triglycerides. These observations raise the question whether Sertoli cells and germ cells are independently affected by the lack of RXRβ or whether one cell type is primarily affected. Abnormal Sertoli cells could be impaired in their ability to correctly assist the maturation and release of spermatids (Redenbach and Vogel 1991; Jegou 1993; Griswold 1995); alternatively, the lipid accumulation in Sertoli cells could result from widespread phagocytosis of abnormal spermatids (Russell et al. 1990).

Three lines of evidence strongly suggest that Sertoli cells may be primarily affected in RXRβ null mutants. First, in wild-type testes, RXRβ appears to be solely expressed in Sertoli cells. Second, at puberty, Sertoli cell abnormalities precede by at least a week the appearance of abnormal spermatids in RXRβ$^{-/-}$ mutants. Lipid droplets were detected in Sertoli cells of 29-day-old mutants, before the completion of the first spermatogenic cycle (Russell et al. 1990 and refs. therein). Therefore, at least the earliest lipid accumulation observed in Sertoli cells cannot be ascribed to the phagocytosis of retained late spermatids. Third, lipid droplets in Sertoli cells were not observed at any stages in testes of RARα mutant mice (Lufkin et al. 1993; Fig. 8a; data not shown) nor in VAD rats (Huang and Marshall 1983) or mice (Fig. 8b; data not shown), even though there is also in these cases a failure of spermatid release accompanied by a phagocytosis of retained spermatids. Thus, lipid accumulation is not a necessary consequence of impaired spermiation. Taken together, these observations suggest that the accumulation of lipids observed in Sertoli cells of RXRβ null mutants reflects a primary metabolic defect.

It is well established that Sertoli cell/germ cell interactions are critical for sperm maturation and spermiation (for review, see Jegou 1993; Griswold 1995). Each maturing spermatid is encased by Sertoli cell cytoplasm that is thought to assist its maturation and its release by as yet poorly understood mechanisms. Thus, the defects of spermiogenesis and spermiation in RXRβ$^{-/-}$ mutant testes could reflect an altered function of Sertoli cells. The occurrence of large lipid droplets within Sertoli cells has been described in several pathological conditions associated with defective spermatogenesis, such as cryptorchidism (Fleeger et al. 1968; for review, see Johnson 1970) or androgen insensitivity (the tfm mice; Chung and Hamilton 1975). Furthermore, high levels of lipids are frequently observed in the testes in cases of human sterility (Johnson 1970 and refs. therein). These observations, together with those made here for RXRβ null mu-

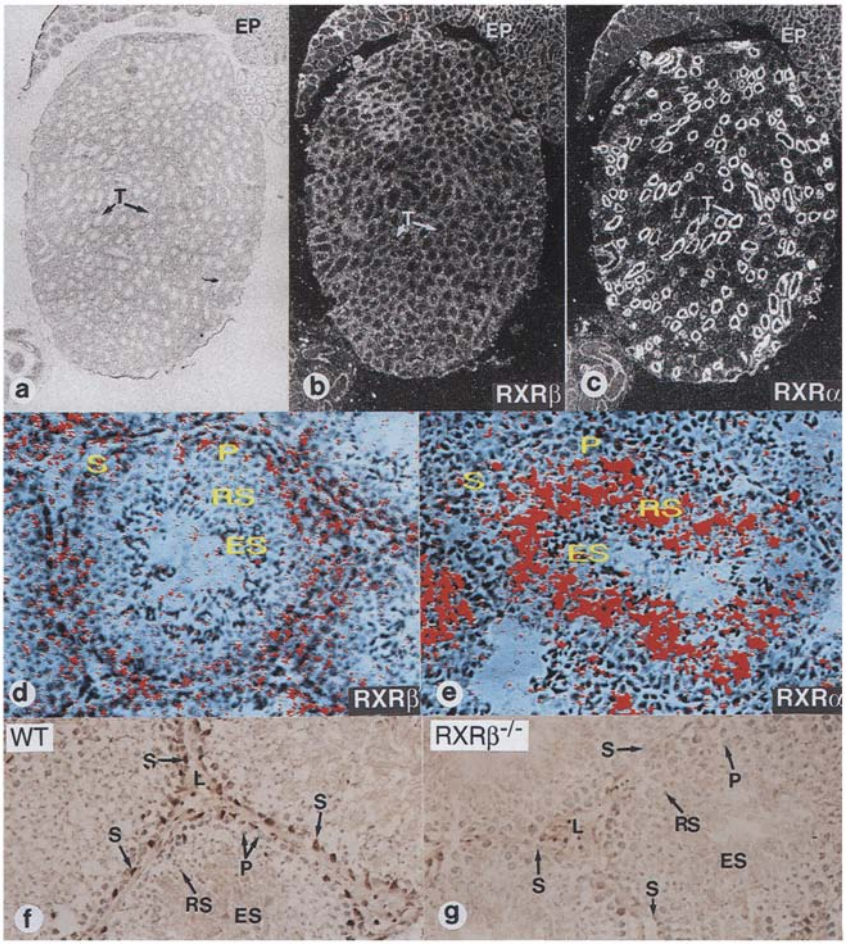

Figure 7. Comparison of the localization of RXRβ (*b,d*) and RXRα (*c,e*) transcripts in a wild-type adult testis and immunoperoxidase staining with an antibody directed against RXRβ of wild-type (WT; *f*) and RXRβ$^{-/-}$ (*g*) testis. (*a*) Bright field; (*b,c*) dark fields. In *d* and *e*, the in situ hybridization signal is shown in false colors after computer processing of a bright-field view and dark-field view of the same section (see Vonesch et al. 1994 for further details). In *f* and *g*, a strong positive signal is exclusively detected in wild-type Sertoli cells. Immunostaining is absent from wild-type germ cells and from mutant Sertoli cells and germ cells. The weak staining of the mutant and wild-type Leydig cells (L) corresponds to background staining because it can be observed even when omitting the anti-RXRβ antibody in the immunostaining sequence. (EP) Epididymis; (T) seminiferous tubules; (S) Sertoli cells or spermatogonia; (P) pachytene spermatocytes; (RS), round spermatids; (ES) elongated spermatids. Magnification, 6× (*a–c*); 95× (*d–g*).

tants, suggest that a faulty lipid metabolism in Sertoli cells could lead to defective spermatogenesis.

The physiological role of lipids during spermatogenesis is unknown. Lipid catabolism might be required solely to generate the energy needed during the transformation of spermatids into spermatozoa and/or their release into the lumen of the seminiferous tubule. Alternatively, some lipids may perform more specific functions as signaling molecules and/or cofactors. In this respect, it is noteworthy that PERF15, the major component of the perinuclear theca (the structure involved in anchoring the acrosomal membrane to the nuclear envelope), has been characterized recently as a homolog of fatty acid-binding protein (FABP) and adipocyte lipid-binding protein (Oko and Morales 1994). If a lipid ligand is indeed required for the function of this protein, the unavailability of such a ligand in RXRβ null mutants could contribute to the detachment of the acrosomal membrane from the nuclear envelope seen in the spermatozoa of these mutants.

Which signaling pathway is affected in RXRβ$^{-/-}$ mutant testes?

The defects observed in RXRβ$^{-/-}$ testes could reflect a requirement of 9C-RA for spermatogenesis and/or the participation of RXRβ as a partner for another nuclear receptor that requires heterodimerization with RXRs to bind to the response elements of its cognate target genes. The roles of retinoids in the testis have been documented from studies of VAD animals (for review, see Eskild and Hansson 1994). VAD testes are characterized by a rapid degeneration of the seminiferous epithelium, because almost all the germ cells are lost within the first 8 days following the initial weight loss (Eskild and Hansson 1994; see also Fig. 8b). It is therefore noteworthy that the RXRβ$^{-/-}$ seminiferous tubules keep their normal cellular architecture at least until 6 months of age. Thus, the function of retinoids in the maintenance of the structure of the seminiferous epithelium does not appear to be precociously impaired in RXRβ$^{-/-}$ mutants. Furthermore, extensive lipid accumulation has not been reported in the Sertoli cells of VAD animals (see also Fig. 8b), suggesting that the defect in lipid metabolism seen in RXRβ$^{-/-}$ mice does not reflect the existence of a 9C-RA-dependent step in this metabolism. The testicular phenotype of RARα null mutants matches closely the lesions resulting from VAD (Lufkin et al. 1993; Fig. 8) and is clearly different from the RXRβ$^{-/-}$ mutant phenotype, because testicular degeneration can occur at

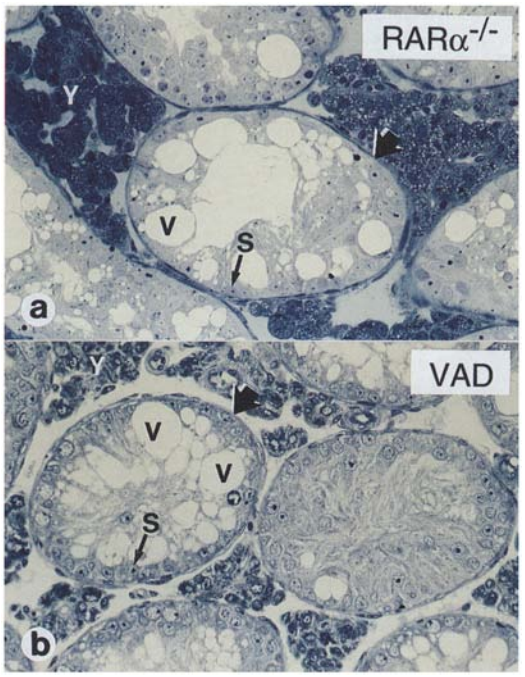

Figure 8. Comparison of semithin sections from osmium-fixed, resin-embedded testes of the seminiferous tubules of 6-month-old RARα$^{-/-}$ mutant (*a*) and VAD (*b*) mice. The VAD males were the F1 offsprings of dams fed a VAD diet and raised on a VAD diet from the time of weaning (S. Ward, in prep.). The large arrows point to similar tubules having lost their germ cells and thus containing only Sertoli cells (S); the cytoplasm of the Sertoli cells contains large vacuoles (V) that are devoid of lipids. (Y) Leydig cells. Magnification, 260×.

much earlier stages in RARα mutants (i.e., before the completion of puberty) in the absence of lipid droplet accumulation (P. Gorry, M. Mark, and P. Chambon, unpubl.). Thus, the retinoid signal that is necessary for spermatogenesis appears to be transduced by RARα, which is expressed in Sertoli cells and spermatids (Eskild et al. 1991), and, if one assumes that RARα requires a RXR partner for its function in seminiferous epithelium, this partner is most probably RXRα. Whether the function of RXRβ in the testis involves in any way the transduction of a retinoid signal will ultimately require the generation of mouse lines bearing mutations that selectively affect the 9C–RA-dependent function(s) of RXRβ.

Members of the PPAR superfamily have been implicated recently as transcriptional regulators of several genes involved in lipid metabolism and have been shown to be activated by various fatty acids (for review, see Desvergne and Wahli 1995). The accumulation of lipids in RXRβ$^{-/-}$ mutant Sertoli cells could therefore reflect an impaired PPAR function. RXRβ may act as a heterodimeric partner for PPARβ, which appears to be highly expressed in Sertoli cells (Braissant et al. 1996). Whether RXRβ could act as a 9C-RA-independent heterodimeric PPAR partner in the testis will require a genetic analysis of PPAR function in the testis and the generation of compound RXRβ/PPARs mutants. In this respect, we note that PPARα mutant mice have been produced recently, that do not exhibit testicular defects (Lee et al. 1995).

Because RXRβ is the most abundant (if not the only) RXR expressed in Sertoli cells, all nuclear receptors that require RXR as a dimerization partner might be functionally impaired in these cells. The present alteration of the lipid metabolism may reflect the impairment of just one of these pathways (possibly the PPAR pathway), and other biochemical defects not detectable at the cytological level may exist in RXRβ null mutant Sertoli cells. Thus, the lipid metabolism defect may not necessarily be the primary cause (or the only cause) responsible for the impaired spermatogenesis. In this respect, we note that in the frog *Rana nigromaculata*, a steroid hormone, 17α,20α-dihydroxy-4-pregnen-3-one, induces spermiation (Kobayashi et al. 1993). If a similar molecule is required for mammalian spermiation and if its effect is transduced by a RXR partner, then the lack of RXRβ could lead to the defect in spermiation observed in RXRβ$^{-/-}$ mutants.

Functional redundancy among RXRs

The defect observed in RXRβ null mice is surprisingly mild in view of the pleiotropic role that has been postulated for RXRs, either as heterodimeric partners for several nuclear receptors and/or as receptors for 9C-RA (see introductory section for references). Because RXRα and RXRβ appear to be widely expressed during embryogenesis and in adult tissues, it is possible that RXRα may functionally compensate for the loss of RXRβ in most instances. In this respect, the complete penetrance, as well as the absence of variation in its expressivity, of the lipid metabolism defect observed in Sertoli cells of RXRβ null mutants, may correspond to a case in which such a redundancy cannot operate, given the apparent absence of RXRα expression in Sertoli cells. On the other hand, the partial penetrance of the embryonic lethal phenotype of RXRβ mutants could result from a partial functional redundancy amongst RXRs. Interestingly, the developmental phenotype of RXRα null mutants affects also a limited set of structures (the heart and the eye; see Kastner et al. 1994a; Sucov et al. 1994). Moreover, the heart malformations seen in RXRα$^{-/-}$ embryos vary greatly in their severity from mutant to mutant, and RXRα/RXRβ double null mutants display much more severe developmental abnormalities than RXRα$^{-/-}$ single mutants (P. Kastner and P. Chambon, unpubl.). In particular, their early cardiac defects are much more severe than those presented by RXRα single mutants. Thus, as observed previously for the RARs (Lohnes et al. 1994; Mendelsohn et al. 1994), the RXRs can be functionally redundant, because RXRβ appears to be able to substitute for the lack of RXRα in many developmental instances.

Conclusion

The RXRβ mutant mice described here offer a new model for further understanding spermatogenesis. In particular, they should provide valuable tools to study the role of Sertoli cells in germ cell maturation and spermiation. Because lipid accumulation has also been observed in the Sertoli cells of human patients with fertility problems, our results raise the possibility that RXRβ mutations may be involved in some cases of human male sterility. Finally, these mice, together with mice mutated in other RXRs or putative RXR heterodimeric partners, will be useful to study genetically the physiological role of RXRs and of their interaction with other members of the nuclear receptor superfamily.

Materials and methods

Targeting vectors and homologous recombination

Genomic clones for the mouse RXRβ (mRXRβ) locus were obtained by screening a genomic library established in λEMBL3 from 129/Sv mouse DNA with an mRXRβ cDNA probe. The structure of the mRXRβ locus was identical to that reported by Nagata et al. (1994). To construct the targeting vector, a 10-kb *Not*I–*Xho*I fragment containing the entire RXRβ locus was inserted into Bluescript (SK−). A *Hin*dIII site was then created by site-directed mutagenesis in exon 3 at the boundary between the B and C regions. Subsequently, the 1.5-kb *Hin*dIII–*Eco*RI fragment containing sequences encoding the entire RXRβ DNA-binding domain was removed and replaced with a PGK–NEO(A^+) cassette (derived from pKJ-1; Adra et al. 1987), which was cloned in antisense orientation. This plasmid, pHR(RXRβ) was linearized with *Xho*I and electroporated into D3 ES cells as described previously (Lufkin et al. 1991). After selection with G418, resistant clones were expanded and genomic DNA was prepared, restricted with *Spe*I, and analyzed by Southern blotting with probe A (Fig. 1a). Three positive clones were obtained that were confirmed with *Hin*dIII or *Kpn*I digests using probe A, as well as a NEO probe. All three clones were injected into C57BL/6 blastocysts, and chimeras derived from two clones (HA9 and HA67) transmitted the mutant allele through their germ line. Routine genotyping of the mice was subsequently performed on *Bam*HI-restricted DNA using probe B (Fig. 1a,b).

Histological procedures

Sections (7 μm thick) from Bouin-fixed, paraffin-embedded testes were dewaxed and stained either with Groat's hematoxylin and Mallory's trichrome (Mark et al. 1993) or by the PAS reaction [the Shiff's reagent was made from pararosaniline (C.I. no. 42500; Sigma) according to Longley's method (Gabe 1968)] followed by a 3-min counterstain with Harris hematoxylin solution (Merck).

Histochemistry, immunohistochemistry, and in situ hybridyzation on frozen tissues

Sections (10 μm thick) from unfixed frozen tissues were collected on poly-L-lysine-coated slides. The sections were (1) hydrated for 3 min in PBS (prior to staining with oil red O or with osmium tetroxyde), (2) fixed for 10 min in 4% paraformaldehyde in PBS at 24°C (prior to the demonstration of acid phosphatase activity), (3) fixed for 3 min in 100% acetone at −20°C, then air-dried (prior to anti-phospholipid immunostaining), or (4) fixed in Zamboni's fixature for 15 min, then treated with 1% H_2O_2 to block endogenous peroxidase (prior to staining with the anti-RXRβ antibody). Staining with the lipid-soluble dye oil red O (C.I. no. 26125; Sigma) in isopropanol/water for 3 min was performed as indicated by Lillie (1965); the sections were counterstained with Harris hematoxylin (5 sec), rinsed in tap water (3 min), then mounted in glycerol–PBS (9:1). Oxidation of unsaturated lipids by osmium tetroxide was achieved by covering the sections with a drop of this fixative (1% in PBS) for 5 min; after rinsing in distilled water, the sections were counterstained with 1% methyl green (C.I. no. 42590; Sigma; purified by chloroform extraction) and mounted in glycerol–PBS. The detection of cholesterol was performed according to Hershberger's modification of the Schultz method (Lillie 1965). The demonstration of acid phosphatase activity in situ was performed according to Burstone, as reported by Lillie (1965): paraformaldehyde-fixed tissue sections were incubated for 2 hr at 24°C in 0.1 M sodium acetate buffer (pH 5.2) containing the substrate naphthol AS-BI phosphate (Sigma) and the diazonium salt fast red violet LB (Sigma). A monoclonal antibody against phosphatidylcholine (MC22-33F; Mark et al. 1992) was used to immunolocalize phospholipid-containing cytoplasmic inclusions on acetone-fixed tissue sections, as described previously. Sections from an adult mouse ovary and the replacement of MC22-33F by nonimmune rat IgM served as positive and negative controls of the immunostaining procedure. Immunostaining with an anti-RXRβ antibody (Sugawara et al. 1995; 1/4000 diluted in TBST) was performed using the ABC Elite system (Vector, Burlingame, CA) according to the manufacturer's instructions. Sections of $RXR^{-/-}$ testis reacted with the anti-RXRβ antibody served as negative controls.

The RXR probes used for in situ hybridization were synthesized from cDNA fragments covering the entire open reading frame (Dollé et al. 1994). In situ hybridization was performed on 10-μm frozen sections as described (Décimo et al. 1996), and the sections were stained with hematoxylin.

Electron microscopy

Mice were perfused intracardially with a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Testes and epididymides were removed and immersed in the same fixative overnight at 4°C. They were then rinsed with this buffer, postfixed for 1 hr at 4°C in cacodylate-buffered osmium tetroxide, dehydrated with graded alcohols, and embedded in epon. For light microscopy, semithin sections (1 μm thick) were stained with toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips 208 electron microscope at 80 kV.

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