Retinoid X receptor beta (RXRB) expression in Sertoli cells controls cholesterol homeostasis and spermatiation

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Abstract

Somatic, targeted inactivation of the retinoid X receptor beta gene (Rxrb) in Sertoli cells (SC; yielding RxrbSer−/− mutants) leads to failure of spermatid release, accumulation of cholesterol esters and, subsequently, testis degeneration. These abnormalities are identical, in their nature and kinetics, to those observed upon inactivating Rxrb in the whole organism, thereby demonstrating that all reproductive functions of RXRB are carried out in SC. The RxrbSer−/− testis degeneration is a consequence of a cholesterol ester cell overload occurring in SC in response to reduced ABCA1- and SCARB1-mediated cholesterol efflux. The failure of spermatiation was also reported in mice lacking the retinoic acid (RA) receptor-α (RARA) in SC (RaraSer−/− mutants) and represents, in addition, a feature of vitamin A deficiency that can be readily induced in mice lacking the lecithin:retinol acyltransferase (Lrta−/− mutants). Altogether, these findings support the conclusion that RXRB heterodimerized with a RA-ligated RARA transduces signals required in SC for spermatid release.


Introduction

Retinoid X receptors (RXRA, RXRB and RXRG) can heterodimerize with a large variety of nuclear receptors (Szanto et al. 2004). Accordingly, Rxa loss-of-function mutations have pleiotropic effects on embryogenesis (Kastner et al. 1997, Mascrez et al. 1998, 2001, Mark et al. 2006) as well as on homeostasis of adult tissues (Li et al. 2000, 2001, 2005, Imai et al. 2001, 2004, Calleja et al. 2006). By contrast, inactivation of Rxrb in the germline (yielding Rxrb−/− mutants, lacking RXRB in the whole organism) results in morphological abnormalities that are restricted to the seminiferous epithelium, namely failure of spermatid release and accumulation of lipids in Sertoli cells (SC; Kastner et al. 1996). Genetic studies in the mouse (Rxrbfl/fl mutants) have also established that the ligand-dependent transcriptional activation function (AF-2) of RXRB is dispensable for spermatiation, while this AF-2 is required in RXRB/LXRB heterodimers to control cholesterol efflux from SC through regulating expression of the membrane-associated ABCA1 transporter (Mascrez et al. 2004). These observations and the fact that vitamin A deficiency does not yield lipid metabolism defects (Ghyselinck et al. 2006) rule out the possibility that retinoic acid (RA) is the ligand activating RXRB in the seminiferous epithelium (Mascrez et al. 2004).

Expression pattern analyses nevertheless left open the possibility that RXRB could exert some functions on testis physiology by acting among in SC (Vernet et al. 2006a), Leydig cells (Gaemers et al. 1998a) or anterior pituitary cells (Krezel et al. 1999). In the present study, we have analyzed the consequences of RXRB ablation only in SC (i.e. in RxrbSer−/− mutants). We have also analyzed the reproductive phenotype of mutant mice carrying null alleles of the lecithin:retinol acyltransferase (LRAT) gene (Lrat−/− mutants; Ruiz et al. 2007) under conditions of dietary vitamin A deficiency. Our data demonstrate that RXRB acts cell autonomously in SC i) to promote cholesterol efflux and ii) to allow proper spermatiation, the process by which the mature spermatids are released into the lumen of the seminiferous tubule. Based on the similarities of the phenotypes displayed by RxrbSer−/− mutants, Rxb−/− mutants (Kastner et al. 1996) and mice lacking the RA-receptor-α (RARA) in SC (RaraSer−/− mutants; Vernet et al. 2006b), we conclude that RXRB/RARA heterodimers, in which RARA is activated by RA, are instrumental to spermatiation.
Results

Failure of spermatiation is a hallmark of the RxrbSer−/− mutation

The testes of 4-month-old RtxrbSer−/− mutants displayed normal germ cell associations (Fig. 1B, D, H and J compare with A, C, G and I). However, numerous mature spermatids did not align at the luminal side of the seminiferous epithelium at stage VII, but remained trapped within the epithelium at stages IX and X (arrowheads in Fig. 1D, compare with C), indicating a severe failure of spermatiation. In addition, all RtxrbSer−/− seminiferous tubules contained TUNEL-positive cellular debris, whose shapes and cyclical variations strongly supported an origin from phagocytosed spermatids (arrowheads in Fig. 1H and J, compare with G and I; Gehin et al., 2002, Mascrez et al., 2004). On the other hand, similarly low amounts of TUNEL-positive spermatocytes were observed in RtxrbSer−/− and wild-type (WT) seminiferous tubules (P*, Fig. 1G and data not shown), thereby excluding the occurrence of massive germ cell death upon ablation of RXRB in SC. Therefore, both histological and TUNEL analyses indicate that the failure of spermatid release and subsequent degradation of

Figure 1 Failure of spermatid release, oligozoospermia and testis degeneration in RtxrbSer−/− mutants. Histological sections of testes (A–J) and epididymides (K and L) at 4 (A–D, G–L) and 12 (E and F) months of age. (A and B) Overview of the seminiferous epithelium prior to the onset of testis degeneration in RtxrbSer−/− mutants; haematoxylin and eosin stain. Note that each ‘hole’ in the RtxrbSer−/− seminiferous epithelium corresponds to a large lipid droplet (L) that was extracted during tissue processing. (C and D) Representative examples of stage IX of the seminiferous epithelium cycle; PAS stain. Note that the presence of numerous retained mature spermatids (arrowheads in D) at the periphery of the RtxrbSer−/− seminiferous epithelium. (E and F) Representative aspects of the seminiferous epithelium in old mice after the completion of testis degeneration in RtxrbSer−/− mutants; toluidine blue stain. The RtxrbSer−/− seminiferous epithelium is restricted to rare SC separated by lipid cholesterol ester crystals. (G–J) TUNEL analysis of WT and RtxrbSer−/− mutant testes prior to the onset of degeneration; the histological sections were counterstained with PAS. In RtxrbSer−/− testes (in H), note that mature (step 16) spermatids at an early phase of their retention (i.e. located at the apical pole of SC and in contact with the lumen of stages VII seminiferous tubules) are TUNEL negative; these spermatids become massively TUNEL positive (arrowheads in J) upon uptake and degradation by SC, suggesting that DNA fragmentation in these cells is a consequence of their degradation by the SC. Normal apoptotic activity, reflected by the occurrence of TUNEL-positive spermatocytes (P* in G), is indistinguishable in RtxrbSer−/− and WT testes. (K and L) Spermatozoa head profiles (Z) are scarce in RtxrbSer−/− epididymis when compared with its WT counterpart; toluidine blue stain. C, cholesterol ester crystals; L, lipid droplets or lipid droplet ‘ghosts’; LP, lamina propria; LU, lumens of seminiferous tubules or epididymis; P*, apoptotic pachytene spermatocyte; SC, Sertoli cell; Sp7, Sp9 and Sp16 refer to steps of spermatid maturation; Z spermatozoa. Roman numerals designate stages of the seminiferous epithelium cycle. Bar (in L): 80 μm (A, B, K and L); 30 μm (C–J); 10 μm (insets in K and L).
Failure of spermiation is a hallmark of an impaired vitamin A signalling pathway

In mice, a state of post-natal vitamin A deficiency is technically difficult to achieve as WT mice have to be born from parents fed a vitamin A deficient (VAD) diet to exhibit pathologies (Van Pelt & de Rooij 1990, Gaemers et al. 1998b; and references therein), and under those circumstances, their seminiferous epithelium may reach some state of vitamin A deficiency before the completion of the first spermatogenic cycle. In contrast to WT mice, mutants carrying null alleles of the gene coding for the blood carrier retinol-binding protein (Rbp4−/− mutants) are unable to mobilize their retinol liver stores (Quadro et al. 1999, Ghyselinck et al. 2006), and mice carrying null alleles of the LRAT gene (Lrat−/− mutants) cannot properly store retinyl esters (Batten et al. 2004, O’Byrne et al. 2005, Ruiz et al. 2007). Therefore, both Rbp4−/− and Lrat−/− mice are much more vulnerable to dietary vitamin A deficiency than are WT mice (Liu & Gudas 2005, Quadro et al. 2005), and represent valuable models to study the VAD status in mice. Intriguingly, the failure of spermiation, an early defect of models to study the VAD status in mice. Intriguingly, the described in solely in SC suffices to recapitulate the oligozoospermia described in Rxb−/− mutants (Kastner et al. 1996).

The failure of spermiation is also consistently observed in mice lacking RARA in SC (Rara−/−/K−/− mutants, Vernet et al. 2006b), thereby strongly suggesting that RXRB/RARA heterodimers are instrumental to spermiation. However, this hypothesis was not genetically validated as we did not find spermiation defects in RxbSer+/−/ RaraSer+/−/K−/−/ mutants (three mutants analyzed). In addition, we did not observe any significant increase in the amount of retained spermatids in RxbSer−/−/ RaraSer−/−/K−/−/ compound mutants (n=3) when compared with the single null mutants (data not shown).

The expression of several candidate genes is altered upon ablation of Rxb in SC

Spermiation is vulnerable to deficiencies in follicle-stimulating hormone (FSH), testosterone or vitamin A (Huang & Marshall 1983, Saito et al. 2000, Beardsley & O’Donnell 2003, Ghyselinck et al. 2006). All of these hormonal signalling pathways act via cell-autonomous regulators of SC functions, namely the FSH receptor (FSHR; Dierich et al. 1998, Abel et al. 2000), the androgen receptor (AR; Chang et al. 2004, De Gendt et al. 2004) and the RA receptor-α (RARA; Vernet et al. 2006b). Quantitative RT coupled to PCR amplification (qRT-PCR) using total RNA extracted from the testes of age-matched (9-week-old) RxbSer−/−/K−/− and WT males (Fig. 3) revealed that the steady-state levels of i) Rara transcripts were not altered in RxbSer−/−/K−/− mutants, ii) Fshhr transcripts were increased by 50%, and iii) Ar transcripts were not altered in RxbSer−/−/K−/− mutants. 

Retained spermatids by SC accounts for the marked decrease in the number of epididymal spermatozoa observed in RxbSer−/−/K−/− mutants (Z, compare with Fig. 1K and L). They also provide evidence that inactivating Rxb solely in SC suffices to recapitulate the oligozoospermia described in Rxb−/− mutants (Kastner et al. 1996).
involved in spermiation. The expression of both genes associated with the SC cytoskeleton and thus potentially

The values (arbitrary units) correspond to the mean amount of

time quantitative RT-PCR analysis for transcripts of the indicated genes.

bar) and wild-type (WT) littermates (grey bar) was subjected to real-

9 weeks of age. Total RNA extracted from testes of

Mtap7 and

Gapdh transcripts, whose expression is not changed upon

***P < 0.001; Student’s t-test). NS, not statistically different.

transcripts exhibited a slight, but significant (P < 0.05) decrease, which is probably physiologically relevant

owing to the fact that SC represent only a fraction of

A majority of seminiferous tubule sections in 8-month-old

RxbSer−/− mutants showed normal germ cell associations, but others displayed a loss of germ cell populations resulting from detachment of healthy immature cells (data not shown, see Kastner et al. 1996). The testes from 12-month-old mutants contained only ‘tubular ghosts’ devoid of all epithelial cells, filled with calcified lipids or with large cholesterol ester crystals and delineated by a thickened, fibrotic lamina propria (compare Fig 1E with F, and data not shown). Thus, seminiferous epithelium degeneration is completed at the same age and exhibits identical morphological features in RxbSer−/− and Rxb−/− mutants (Kastner et al. 1996 and present report).

Fatty degeneration of the testis in old Rxb Ser−/− mutants

A majority of seminiferous tubule sections in 8-month-old RxbSer−/− mutants showed normal germ cell associations, but others displayed a loss of germ cell populations resulting from detachment of healthy immature cells (data not shown, see Kastner et al. 1996). The testes from 12-month-old mutants contained only ‘tubular ghosts’ devoid of all epithelial cells, filled with calcified lipids or with large cholesterol ester crystals and delineated by a thickened, fibrotic lamina propria (compare Fig 1E with F, and data not shown). Thus, seminiferous epithelium degeneration is completed at the same age and exhibits identical morphological features in RxbSer−/− and Rxb−/− mutants (Kastner et al. 1996 and present report).

Progressive accumulation of cholesterol esters and decreased Abca1 and Scarb1 expression in RxbSer−/− testes

Numerous lipid droplets were visible at the periphery of seminiferous tubules in all RxbSer−/− testes, already by the end of puberty (i.e. at 1 month of age, Fig. 4A). They were localized within the cytoplasm of SC, as assessed by electron microscopy (not shown), and they markedly enlarged upon ageing (L, Fig. 4A–C, right panels). By contrast, similar lipid droplets remained small in WT testes (L, Fig. 4A–C, left panels). Thus, with respect to their early appearance, localization and kinetics of enlargement with ageing, the RxbSer−/− lipid droplets are undistinguishable from those of Rxb−/− testes (Kastner et al. 1996).

The lipid droplets in RxbSer−/− testes were stained positively by histochemical procedures detecting neutral fats (L in Fig. 4E, compare with D) and notably cholesterol and its esters (L in Fig. 4G, compare with F). Accordingly, thin layer chromatography (TLC) profiles showed a large excess of cholesterol esters in lipid extracts from RxbSer−/− testes, with no increase in triglyceride levels (Fig. 4L). Therefore, lipids extracted from RxbSer−/− testes contained an increased amount of cholesterol esters as their Rxb−/− counterparts (Mascrez et al. 2004).

We quantified the expression of the mRNA encoding for ABCA1 and SCARB1, which both promote cellular cholesterol efflux from SC (Selva et al. 2004, Duong et al. 2006, and references therein). In 9-week-old RxbSer−/− mutants, the mRNA levels of Abca1 were significantly (P < 0.001) decreased by about 40% (Fig. 3). It is worth noting that in younger (i.e. 5-week-old) mutants, Abca1 expression was even further decreased (not shown).

Thus, ablating Rxb solely in SC induces a decrease in Abca1 expression of the same order of magnitude than that generated by a Rxb loss-of-function mutation in the whole organism (i.e. the germline deletion of the RXRB domain containing the AF-2 function, RxbAb2o mutants; Mascrez et al. 2004). It additionally significantly decreases (P < 0.001) the expression of Scarb1 (Fig. 3). As such a decrease is not observed in RxbAb2o mutants (Mascrez et al. 2004), it appears that a transcriptionally inactive RXRB (i.e. without its AF-2) can control Scarb1 expression in WT SC.

Discussion

RXRB action on spermiation is SC autonomous and mediated by RXRB/RARA heterodimers in which the RAR partner is RA liganded

RxbSer−/− mutants display an impaired spermiation as severe as that of Rxb−/− mutants (Kastner et al. 1996), thus demonstrating that RXRB controls mechanisms that allow proper spermatid disengagement on the SC side. We have previously established that spermiation does not require a transcriptionally active RXRB (Mascrez et al. 2004), and have shown that inactivation either in whole mouse or specifically in SC of potential
heterodimerization partners for RXRB (namely, THRA, THRB, LXRB, PPARA, PPARD or VDR, which are all expressed in SC), does not yield a spermiation failure (Mascrez et al. 2004, and our unpublished results). These observations rule out the involvement, in spermatic release, of a ligand-activated RXRB as well as of heterodimers of RXRB with the nuclear receptors listed above. On the other hand, the occurrence of a spermiation failure in the Lrat<sup>+/−</sup> mouse model of vitamin A deficiency (present report) as well as in the Rbp<sup>+/−</sup> mouse model (Ghyselinck et al. 2006) proves that RA, the RAR ligand, is indispensable for spermatic release. As in addition mutant mice lacking RARA in SC also fail to release spermatids (Vernet et al. 2006b), it appears that RXRB/RARA heterodimers in which RARA is RA liganded play a crucial role in spermiation.

Spermiation becomes reduced upon a modest decrease in AR activity (Holdcraft & Braun 2004). Thus, our demonstration that RXRB promotes the expression of Ar in SC suggests that AR may act in spermatic release downstream of the RA-signalling pathway. In contrast to the AR, the FSHR is probably not involved in spermiation under physiological conditions (Dierich et al. 1998, Abel et al. 2000, Wreford et al. 2001). However, our observation that Fshr expression is increased upon inactivation of Rxb in SC, together with the fact that the Fshr promoter contains a binding site for RAR that mediates repression by RA (Xing & Sairam 2002), supports the view that RXRB/RARA heterodimers may perform additional functions in SC independently from spermiation.

Spermiation involves a variety of cell adhesion molecules and requires the integrity of the SC cytoskeleton (Russell et al. 1989, Fleming et al. 2003, Lee & Cheng 2004). In this context, it is noteworthy that inactivating RXRB specifically in SC i) decreases the expression of genes encoding proteins associated with actin microfilaments (i.e. Rai14; Peng et al. 2000, Kutty et al. 2001) or with microtubules (i.e. Mtap7; Komada et al. 2000) and ii) may disorganize the vimentin network through reducing expression of Ar (Show et al. 2003). On the other hand, expression of espin, a component of SC–spermatid ectoplasmic specialization junctional plaques (Bartles et al. 1996), is not altered in Rxb<sup>Ser−/−</sup> mutant testes (data not shown).

**Figure 4** Kinetics of lipid accumulation and identification of the lipid types present in Rxb<sup>Ser−/−</sup> testes. (A–C) Progressive enlargement of the lipid droplets (black dots) upon ageing in Rxb<sup>Ser−/−</sup> testes; osmium tetroxide stain. (D and E) Histochemical characterization of lipids. The hydrophobic dye, oil red O, stains all neutral lipids, including triglycerides and cholesterol esters (in D), whereas the Romieu’s modification of the Liebermann–Buchard’s reaction stains specifically cholesterol and its esters (in E). Note that in (D), the DAPI nuclear counterstain was converted to a bright field image (blue false colour) and then superimposed with the oil red O staining (red colour) using Photoshop. (F) Thin layer chromatography of lipids extracted from testes of littermates with the indicated genotypes at the age of 5 weeks; each line corresponds to a distinct mouse. Legend: C, cholesterol; CE, cholesterol esters; E, elongate spermatids; L, lipid droplets in Sertoli cells; P, pachytene spermatocytes; R, round spermatids; SC, Sertoli cells; T, seminiferous tubules; TG, triglycerides; Y, Leydig cells. The arrowheads in (D) point to lipid droplets normally present in elongate spermatids and the asterisk in (F) indicates the migration front. Bar (in E): 160 μm (A–C); 20 μm (D–E).
Age-related testis degeneration in RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants results from cholesterol ester accumulation

The size of the SC lipid droplets, which we have followed over a period of 2 years, does not increase in WT mice after sexual maturity (Fig. 4 and data not shown). By contrast, a pronounced enlargement of the lipid droplets that reach the size of SC nuclei between 6 and 8 months of age is observed in RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants. As components of the SC cytoskeleton are essential for the maintenance of the seminiferous epithelium (Lee & Cheng 2004), it is conceivable that these large lipid droplets could mechanically impair organization of SC cytoskeleton, thereby disturbing their adhesion to germ cells and leading to the sloughing of immature spermatids into the lumens of the seminiferous tubules. Additionally or alternatively, it has been proposed that an excess of intracellular cholesterol is toxic through the formation of cholesterol ester crystals, triggering apoptotic pathways, formation of toxic oxysterols and disruption of membrane domains that are crucial for the function of particular enzymes or signalling molecules (Tabas 2002, Cui et al. 2007). The toxicity caused by excess cholesterol could even further promote cholesterol build-up through compromising the mechanism by which cells efflux cholesterol (Feng & Tabas 2002). Thus, over time, cholesterol accumulation could kill SC yielding tubular ghosts devoid of an epithelium. In any event, cholesterol accumulation in SC can, on its own, account for the age-related testis degeneration in RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants.

It is noteworthy that the rate of lipid accumulation in SC is identical between RXRβ<sup>−/−</sup> and RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants (Fig. 4A–C; Kastner et al. 1996), but is slower in RXRβ<sup>Al2α</sup> mutants (Mascrez et al. 2004, and our unpublished data). Accordingly, the completion of testis degeneration, manifested by the disappearance of the seminiferous epithelium, occurs earlier (i.e. by 1 year of age) in RXRβ<sup>−/−</sup> and RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants (Kastner et al. 1996, and present report) than in RXRβ<sup>Al2α</sup> mutants (Mascrez et al. 2004). The necessity for SC to metabolize larger amounts of cholesterol originating from retained mature spermatids in RXRβ<sup>−/−</sup> and RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants and the significant decrease in the expression of Scarb1 in RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants, but not in RXRβ<sup>Al2α</sup> mutants, can both account for these differences.

Materials and Methods

Mice

All mice, with a mixed C57BL/6–129/Sv (50%–50%) genetic background, were housed in an animal facility licensed by the French Ministry of Agriculture (agreement N°B67-218-5) and all animal experiments were supervised by NBG (agreement N°67-205), in compliance with the European legislation on care and use of laboratory animals. The breeding diet (D03) contained 25 000 UI of vitamin A per kg (UAR, Villemoisson sur Orge, France). The VAD diet (TD86143) was purchased from Harlan (Gannat, France) and provided ad libitum. Food consumption was identical between WT and mutants (not shown). For depletion studies, mice (n = 4 in each group) were fed the breeding diet from weaning to 6 weeks of age, and then the VAD diet for the indicated number of weeks.

Mice bearing loxP-flanked (floxed) RXRβ gene (RXRβ<sup>Al2α</sup>; Li et al. 2005) and Amh-Cre transgenic mice (Amh-Cre<sup>00</sup>; Lécureuil et al. 2002) were genotyped as described. To specifically inactivate RXRβ gene in SC, females carrying two floxed alleles of RXRβ (i.e. RXRβ<sup>Al2α</sup> females) were crossed with males bearing both the Amh-Cre transgene and one floxed allele of RXRβ (i.e. Amh-Cre<sup>00</sup>/RXRβ<sup>Al2α</sup> males). These crosses generated males in which RXRβ was inactivated in SC (i.e. Amh-Cre<sup>00</sup>/RXRβ<sup>Al2α</sup>); these mice were referred to as RXRβ<sup>Ser<sup>−/−</sup> </sup> mutants. They also generated RXRβ<sup>Al2α</sup> and RXRβ<sup>Al2α</sup> control males, which did not display histological defects and were referred to as WT mice. Mice lacking LRAT (Lrat<sup>−/−</sup>) mutants were characterized previously. They were genotyped as described (Ruiz et al. 2007).

Table 1 Sequence of primers used for quantitative RT-PCR amplifications.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Primers (forward and reverse)</th>
<th>Position (nt)</th>
<th>Size (nt)</th>
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<tr>
<td>Abca1</td>
<td>NM_013454</td>
<td>5'-AGCCAGAAGGGATGTCAAG-3', 5'-ATGCGATCTGGTGAACCT-3'</td>
<td>2955–2974</td>
<td>102</td>
</tr>
<tr>
<td>Ar</td>
<td>NM_013476</td>
<td>5'-GGCAATGTGTTTACCCATCG-3', 5'-TCGTTTTCTGTCGGCAGT-3'</td>
<td>1601–1620</td>
<td>171</td>
</tr>
<tr>
<td>Fshr</td>
<td>NM_013523</td>
<td>5'-TTGCCCTGATGATGTTTCTCAG-3', 5'-CTGCCCTCAACTCTCTTCG-3'</td>
<td>688–707</td>
<td>116</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_008084</td>
<td>5'-CAAGGTCCACCCGACACTTGG-3', 5'-GAGCCATCTCGCTTCCGG-3'</td>
<td>527–549</td>
<td>89</td>
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<tr>
<td>Mtap7</td>
<td>NM_008635</td>
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<td>615–596</td>
<td>125</td>
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<tr>
<td>Rai4</td>
<td>NM_030690</td>
<td>5'-GGATGTGCCGCGACGGAT-3', 5'-CCCGGGACTGTCGATGATG-3'</td>
<td>1470–1489</td>
<td>125</td>
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<tr>
<td>Rara</td>
<td>NM_009024</td>
<td>5'-CCGCTCATCCAGCTAGGTTA-3', 5'-AGCCGGACCTTGGTCAATG-3'</td>
<td>695–715</td>
<td>59</td>
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<tr>
<td>Scarb1</td>
<td>NM_016741</td>
<td>5'-CTTGACACCCGAGACTT-3', 5'-CACCCTTGGCAGAGATAT-3'</td>
<td>984–1003</td>
<td>142</td>
</tr>
</tbody>
</table>

The gene names, the accession numbers, the forward (upper line) and reverse (lower line) primers, their positions in the sequences and the sizes of the amplified fragments are indicated. nt, nucleotide.
Histology, histochemistry and TUNEL assays

Histological observations were repeated on three to four males per genotype and age group. Staining of histological sections with haematoxylin and eosin, periodic acid Schiff (PAS), osmium tetroxide, toluidine blue and oil red O, and histochemical detection of cholesterol esters were as described (Mark et al. 2007). For detection of apoptotic cells (TUNEL assays), testis samples were fixed in 4% (w/v) paraformaldehyde in PBS for 16 h at 4 °C, then embedded in paraffin. TUNEL-positive cells were detected using the In Situ Cell Death Detection Kit, peroxidase (Roche), and the sections were counterstained with PAS.

TLC of lipid extracts

Lipids from testes (three different animals for each genotype) were extracted using the Bligh and Dyer procedure, dissolved in 0.5 ml methanol–chloroform (1:2), loaded onto TLC plates (Machery Nagel, Hoerdt, France) that were run in heptane–diethyl ether–acetic acid (7:2:1) for 5 min, then in heptane for 10 min, and revealed by molybdatophosphoric acid staining. TLC analyses were repeated three times. Standard lipids were from Sigma.

Analysis of RNA

Total RNA was prepared using Trizol reagent (Invitrogen Life Technologies). Quantitative analysis of RNA was carried out by two step RT coupled to quantitative real-time PCR using a Light-Cycler 1.5 (Roche Molecular Biochemicals). Reverse transcription of 2 µg total RNA followed by PCR amplification of cDNAs were performed using QuantiTect Reverse Transcription and QuantiTect SYBR Green PCR Kits respectively, according to the manufacturer’s instructions (Qiagen). Conditions were 45 cycles with denaturation for 15 s at 95 °C, annealing for 15 s at 60 °C and elongation for 15 s at 72 °C. Each cDNA sample was tested in triplicate. The transcript levels were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase transcripts. Analysis of RNA could be perceived as prejudicing the impartiality of the research reported.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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