AKR1B7 (mouse vas deferens protein) is dispensable for mouse development and reproductive success

C Baumann, B Davies, M Peters¹, U Kaufmann-Reiche¹, M Lessl¹ and F Theuring

Center for Cardiovascular Research/Institute of Pharmacology and Toxicology, Charité University Medicine Berlin, Hessische Strasse 3-4, 10115 Berlin, Germany and ¹Research Laboratories of Bayer Schering Pharma AG, Muellerstrasse 178, 13342 Berlin, Germany

Correspondence should be addressed to F Theuring; Email: franz.theuring@charite.de

C Baumann is now at Department of Clinical Studies, School of Veterinary Medicine, Center for Animal Transgenesis and Germ Cell Research, University of Pennsylvania, Kennett Square, Pennsylvania, USA
B Davies is now at Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK

Abstract

AKR1B7 (aldo-keto reductase family 1, member 7; also known as mouse vas deferens protein) is a member of the AKR superfamily, and has been suggested to play a role in detoxifying processes on account of its preferred substrates, 4-hydroxynonenal and isocaproaldehyde. High levels of protein expression were found in the vas deferens and the adrenal gland, where sustained expression is dependent on androgen or ACTH respectively. Recently, a remarkable induction of AKR1B7 expression has been reported in the ovary following exogenous injections of LH. In the present study, we confirm this regulation physiologically during the estrous cycle, observing *Akr1b7* expression to be restricted to the theca and stromal cells of the proestrus ovary. To further investigate the role of this detoxifying enzyme in both male and female reproduction, we generated knockout mice deficient in AKR1B7. Although AKR1B7 expression in the vas deferens is considerable and tightly regulated in the ovary of wild-type animals, homozygous mutant animals were found to be viable and no reproductive phenotype was observed. Ovarian follicle maturation and spermatozoa parameters remained normal in the absence of this protein. The determination of serum progesterone revealed an increase in hormone concentration in metestrus, while progesterone was found to be decreased in the estrus phase of the cycle in knockout females.

Reproduction (2007) **134** 97–109

Introduction

Aldo-keto reductases (AKRs) are NADPH-dependent oxidoreductases, which convert a broad variety of carbonyl compounds into their corresponding alcohols (Bohren *et al.* 1989). Monosaccharides, steroids, prostaglandins, xenobiotics as well as aliphatic and aromatic aldehydes belong to the preferred substrates (Jez *et al.* 1997). The AKRs comprise a diverse gene family consisting of at least 119 members to date. A multitude of species ranging from higher vertebrates and plants to protozoa, eubacteria, fungi, and archaeobacteria has been found to express AKRs. In accordance with the broad substrate specificity reported for members of this superfamily, the individual enzymes show divergent functional specialization (Hyndman *et al.* 2003).

The initial discovery of AKR1B7 (AKR family 1, member 7; also known as mouse vas deferens protein) as the major protein of the murine vas deferens (Taragnat *et al.* 1986) has been causative for its designation.

Subsequently, a high level of expression was reported in the zona fasciculata of the adrenal cortex (Lau *et al.* 1995, Aigueperse *et al.* 1999) in ovaries following stimulation with a luteinizing hormone (LH) analog (Brockstedt *et al.* 2000) and, to a lesser extent, in several other organs, e.g., kidney, intestine, eye, and seminal vesicle (Lau *et al.* 1995). Extensive investigation of the gene's transcriptional regulation found the vas deferens expression to be androgen-dependent (Taragnat *et al.* 1988, Martinez *et al.* 2001) and similarly, adrenal expression was found to be dependent on corticotrophin (adrenocorticotrophin (ACTH); Lefrançois-Martinez *et al.* 1999, Martinez *et al.* 2001). We previously demonstrated a remarkable 300-fold induction of AKR1B7 protein in ovarian theca cells following application of human chorionic gonadotropin (hCG). AKR1B7 protein is only detectable within a timeframe of 1.5–14 h after hCG treatment, thus suggesting an important role for this AKR in ovulatory processes (Brockstedt *et al.* 2000). The promoter region of

Akr1b7 has been shown to contain two sequence motives with high homology to the cAMP response element (Aigueperse *et al.* 1999). The rapid induction of *Akr1b7* mRNA within 1.5 h post-hCG might, therefore, be a direct effect of cAMP on the promoter (ACTH as well as LH exert their actions mainly via cAMP). Moreover, expression of *Akr1b7* in the adrenal gland is dependent on transcription factors like NR5A1 (also known as SF1) and CCAAT/enhancer binding protein (C/EBP)- β (Aigueperse *et al.* 1999), which are additionally involved in the regulation of expression of steroidogenic genes.

A regulation by the LH/hCG surge has also been shown for cytochrome P450scc (cholesterol side-chain cleavage enzyme, *Cyp11a1*) mRNA (fivefold increase within 7 h) in theca and granulosa cells (Goldring *et al.* 1987, Richards *et al.* 1987). Taken together, *Akr1b7* expression coincides with the induction of cytochrome P450scc (*Cyp11a1*) mRNA expression in theca cells and in the adrenal gland and is regulated by inducers of steroidogenic activity.

In vitro analysis of substrate specificity revealed a preference of AKR1B7 protein for isocaproaldehyde as well as for 4-hydroxynonenal (4-HNE; Lefrançois-Martinez *et al.* 1999), two highly toxic and harmful compounds (Dianzani *et al.* 1999, Pizzimenti *et al.* 2002) whose detoxification into less reactive metabolites is of considerable importance for cellular survival. Isocaproaldehyde accumulates as a byproduct in one of the first steps of steroidogenesis during cholesterol side-chain cleavage (Constantopoulos *et al.* 1966, Burstein & Gut 1971, Esterbauer *et al.* 1991). In concordance with its substrate specificity, the adrenal and ovarian sites of AKR1B7 expression are important sources of steroidogenic hormones such as glucocorticoids, progestins and androgens, and thus a role in detoxifying isocaproaldehyde in these tissues is hypothesized. Moreover, *Akr1b7* expression could not be detected in granulosa cells, the corpus luteum, or the placenta. The vas deferens in contrast is not known to be steroidogenic; however, here *Akr1b7* expression is exceptionally high. Whether the protein fulfills several distinct functions depending on the expressing cell type or organ is not known. A general function as a detoxifying protein in steroidogenesis is indeed unlikely; however, several lines of evidence suggest such a role in theca cells of the ovary and in the adrenal gland.

The formation of the highly reactive 4-HNE occurs through reactive oxygen species-induced peroxidation of polyunsaturated fatty acids (Yi *et al.* 1997, Comporti 1998). Since the membranes of spermatozoa, transported by the vas deferens, contain higher concentrations of polyunsaturated fatty acids in comparison with other cell types (Aitken 1994), the remarkable strong expression of AKR1B7 within this organ may represent a protective mechanism against this toxic aldehyde, ensuring sperm viability.

The identification of AKR1B7 as a potent enzyme catalyzing 4-HNE as well as isocaproaldehyde *in vitro* together with its spatial and temporal expression pattern within the ovary, vas deferens, and furthermore within the adrenal gland suggests a potential physiological role for this enzyme in reproductive function. Using the Cre/loxP technology, we prepared knockout animals (*Akr1b7*^{tm1.1Fth}/*Akr1b7*^{tm1.1Fth}) and ascertained the effect of targeted deletion of the *Akr1b7* gene on reproductive physiology.

Materials and Methods

Animals

Mice used throughout this study were kept under controlled temperature (20–22 °C), with a 12 h light:12 h darkness cycle, relative humidity of 50–70%, and food and water *ad libitum*. All animal studies were performed in compliance with the Animal Care and Use Committee guidelines of the Land Berlin, Germany.

In situ hybridization

Vaginal cytology was used for the determination of the estrus cycle according to the method described by Butcher *et al.* (1974). LH concentrations in proestrus reach maxima (LH surge) at ~1800 h (Bailey 1987). Therefore, vaginal smears were obtained daily at 1800 h over a period of 20 days and, immediately after that tissues were collected from animals at diestrus, proestrus, estrus, and metestrus. At proestrus stage, tissue preparation was additionally conducted 6 and 14 h post-assumed LH surge. mRNA expression was exclusively assessed on ovaries from animals showing at least three consecutive 4-day estrous cycles.

Localization of *Akr1b7* mRNA in tissue sections was performed by non-radioactive *in situ* hybridization. Tissue fixation, template preparation, *in situ* hybridization, and immunological detection were conducted as described earlier (Brockstedt *et al.* 2000).

Gene targeting in embryonic stem (ES) cells

Mouse genomic PAC clones containing the murine *Akr1b7* gene were obtained from a 129 Ola mouse genomic library (German Resource Center GmbH, Berlin, Germany) using a mouse cDNA probe corresponding to the exons 2–4 of the *Akr1b7* gene. A 3 kb *Dra*I genomic fragment corresponding to exons 2–4 was cloned into pBluescript KS (Stratagene) 3' of a previously inserted loxP sequence, creating the plasmid pAKR1B7loxP. The 5' homology arm, a 2.4 kb genomic fragment corresponding to a region spanning intron 1, was amplified by PCR (Expand Long Template PCR System, Boehringer Mannheim) using the primers 5'-gAAgCTTAggCCAgCATTATCACCTgTTgTCag-3' and 5'-TAAgCTTgCAATgTgATTgAACTCTGCTAgg-3'

(containing restriction enzyme-cleavage sites for HindIII) and was cloned into the pAKR1B7loxP plasmid, 5' of the loxP site via HindIII, creating the plasmid pAKR1B7loxP5'. The 3 kb 3' homology arm, corresponding to a region containing exons 5–8, was amplified using the primers 5'-TgTCgACAACTCTCCTCCCAATgACTCTCC-3' and 5'-ggTgTAggTGTgCTAAgTATgTATggCCTg-3' (containing restriction enzyme-cleavage sites for Sall and XhoI respectively) and was cloned into a XhoI site of the ploxPneo vector, a plasmid containing a floxed selection cassette, comprising the neomycin phosphotransferase gene under independent promoter control (*Pgk1*) and an SV40 polyadenylation sequence (Yang *et al.* 1998). This floxed cassette together with the 3' targeting arm was then inserted 3' of the Dral fragment creating the plasmid pAKR1B7-KO. The targeting vector was completed by the insertion of two copies of a herpes simplex virus thymidine kinase (*Tk1*) gene, serving as negative selection cassette (Smith 1991; Fig. 2A). The prepared vector was linearized by XhoI and transfected into E14.1 ES cells. The resulting neomycin-/gancyclovir-resistant colonies were screened for homologous recombination within the 5' homologous region by PCR. A 2.3 kb amplification product was obtained from homologous recombined clones using the primers 5'-CagAgCATC-TATCTgCCTTgggTTTgA-3', a forward primer hybridizing to a sequence in exon 1 and 5'-gggTTATTgAATATgATCg-gAATTCgA-3', a reverse primer hybridizing to the loxP site 5' of exons 2–4. Homologous recombination within the 5' and 3' homology arms was confirmed using Southern analysis (data not shown). Three independent clones showing correct homologous recombination were expanded and transiently transfected with a Cre recombinase expression vector (Taniguchi & Hayashi 1998) in order to remove the selection marker together with the genomic region corresponding to exons 2–4. Analysis of G418-sensitive clones yielded a variety of recombined clones suitable for the generation of a constitutive knockout (Type III) as well as for the generation of a floxed allele (Type I; deletion of the selection cassette alone). Screening of the resulting clones was performed by Southern analysis. A PCR generated probe, corresponding to the 5' region of intron 1, specifically detected fragments of 11 kb (wild type) and 9 kb (Type III recombined) within BglII digested genomic DNA.

Generation of knockout mice

Type III recombined ES cell clones were injected into the blastocysts of C57BL/6 mice and the resulting chimeric males were mated to C57BL/6 females. Genotyping of the progeny was performed by PCR analysis of tail-tip DNA using a forward primer hybridizing to intron 1 (5'-TgC CTC AgC CTC TCA CA-3') and two reverse primers, one hybridizing to the floxed region, directly upstream of exon 2 which is deleted in the knockout allele (Type III recombined; 5' gCA ggg CCC ATA ACT Tg 3') and the other hybridizing downstream of exon 4 (5' TTg ggA gAA gAA AAC ATA CAC T 3'). The progeny of heterozygous matings

was subjected to confirmatory Southern analysis according to the strategy described earlier. The resulting knockout allele has the designation *Akr1b7^{tm1.1Fth}* (MGI: 3629297).

Northern and western analysis

Total RNA, isolated from freshly prepared vasa deferentia, adrenal glands, and ovaries (8 h post-hCG stimulation) using Trizol (Invitrogen Life Technologies), was electrophoretically separated on a 1% agarose gel in the presence of 18% formaldehyde and transferred to Hybond N membranes (Amersham). Hybridization according to standard conditions was performed with a ³²P-radiolabeled probe corresponding to nucleotides 843–1183 (exons 9 and 10) of the submitted *Akr1b7* sequence (Accession number: J05663). Immature mice at 3 weeks of age were injected intraperitoneally with a single dose of 20 IU follicle-stimulating hormone (Intergonan, Intervet, Unterschleissheim, Germany) and 48 h later, with 10 IU hCG (Ovogest, Intervet) to robustly induce *Akr1b7* expression.

Expression analysis of *Akr1b8* by northern blotting in adrenal glands and vasa deferentia was conducted using a *Akr1b8*-specific cDNA probe, spanning a region in the 3'UTR between 1049 and 1272 bp of the submitted *Akr1b8* sequence (Accession number: U04204).

For western analysis, a synthetic peptide antigen comprising the amino acid sequence of murine AKR1B7 between residues 121 and 141 (ALLPKDNKGVLLSKSTFLDA) was coupled to keyhole limpet hemocyanin via an additional cysteine at the carboxy terminus (Schnolzer *et al.* 1992) and was used to produce a polyclonal antiserum in rabbits. Tissue samples from vas deferens, adrenal glands, and ovaries 8 h following an ovulatory hCG injection were pulverized in liquid nitrogen and resuspended in 250 mM Tris-HCl (pH 7.4), 0.4 mM phenylmethylsulfonyl fluoride. Soluble tissue extracts (20 µg) were subjected to SDS-PAGE on 12% acrylamide gels for electrophoretic separation, according to Laemmli (1970) and subsequently transferred to PVDF membranes (Bio-Rad). The membranes were incubated for 1 h at room temperature in Tris-buffered saline solution containing 5% skimmed milk and incubated over night at 4 °C with AKR1B7-specific antipeptide antiserum (1:1000) recognizing an epitope within the deletion domain. Peroxidase-conjugated anti-rabbit IgG secondary antibodies were added at a 1:2500 for 1 h at room temperature. Peroxidase activity was detected with the ECL System (ECL, Amersham Pharmacia Biotech).

Histology, organ weight determination, and assessment of follicle maturation stages

Organ weight of the ovaries, testes, epididymides, and adrenal glands was determined immediately after dissection from the body cavity and removal of connective tissue.

Animals used were 20 weeks of age. Tissues were then fixed for 24 h in 4% PBS-buffered paraformaldehyde for histological analysis. After dehydration, paraffin embedding and sectioning, hematoxylin and eosin staining were performed according to standard procedures. In order to evaluate the distribution pattern of different follicle-maturation stages, 5 µm serial sections of the entire organ of knockout animals ($n=4$) and wild-type littermates ($n=4$) at metestrus phase of the cycle were prepared. Evaluation of follicle maturation stages in serial sections was restricted to every fourth section using the nucleus of the oocyte as a guide. Under the assumption of a 20 µm diameter of the nucleus, this procedure was chosen to avoid double evaluation of single follicles. This principle was also adhered to for the evaluation of atretic follicles. Only follicles containing an intact oocyte with a nucleus were considered, and follicles with fragmented oocytes and disrupted nuclei were eliminated. The presence of pyknotic granulosa cell nuclei (shriveled cells with fragmented dark nuclei and apoptotic bodies) and/or the degeneration of the oocyte nucleus served as criteria of atresia. Since morphological signs of atresia were not observed in follicles from stage 3 to 4, the data presented refer exclusively to follicles of stages 5–8. Follicle maturation stages were classified according to [Pedersen & Peters \(1968\)](#) and [Hegele-Hartung et al. \(2004\)](#). Type 3a and 3b follicles are characterized by a single cell layer of granulosa cells surrounding the oocyte, whereas type 4 follicles comprise oocytes with two layers of granulosa cells. Type 5a and 5b follicle show several layer of granulosa cells around the oocyte and are classified as preantral follicles. The beginning of the antrum formation marks type 6 follicles, followed by further advanced stages of antral follicles (type 7 and type 8).

Cumulative estrous cycle staging

For the analysis of estrous cycle stage durations, ovaries were collected from 6- to 10-week-old knockout ($n=12$) and wild-type ($n=12$) females immediately after assessment of the estrous cycle stage (daily between 1700 and 1800 h). Classification was performed according to [Rugh \(1968\)](#). Accordingly, proestrus vaginal smears contain mainly epithelial cells that are round and visibly nucleated. Cells from the estrus phase are cornified squamous epithelial cells with a highly granular cytoplasm and an irregular shape that retain a visible nucleus in early estrus; however, they lack a visible nucleus in the later estrus stage of the cycle. Small leukocytes with granular cytoplasm predominate in the metestrus and diestrus phases.

Fertility studies

To compare fertility parameters of knockout females ($n=5$ –10/age group) versus wild-type littermate controls ($n=5$ –6/age group), animals at 30 days of age (age

group 1) or at 5 months of age (age group 2) were mated with 10-week-old male C57BL/6 animals. The second age group was chosen to increase the number of 4- to 5-day estrous cycles experienced before the first pregnancy, to assess whether continuous pregnancy beginning immediately after puberty might mask an effect of the gene deletion. Knockout males ($n=10$) and wild-type littermate controls ($n=13$) which are 6-week-old were mated with the 8-week-old NMRI (outbred Swiss mouse, Naval Medical Research Institute, USA) females. All matings were performed at a ratio of 1:1. Breeding pairs were maintained together for a period of 10–15 months and successful mating was determined by the observation of a vaginal plug. Breeding efficiency was determined by analysis of average litter sizes and frequencies of parturition.

Analysis of spermatozoa

Spermatozoa were collected unilaterally from the cauda epididymis and the vas deferens respectively of knockout ($n=8$) and wild-type littermate control ($n=10$) animals at the age of 22 weeks. The cauda region of the epididymis and the entire vas deferens were cut open and incubated for 10 min in prewarmed PBS at 37 °C to allow the spermatozoa to emerge as described previously ([Davies et al. 2004](#)). A homogenous suspension was then diluted, spread over a microscope slide, and spermatozoa were counted and assessed. The motility was defined as the percentage of motile spermatozoa present in a defined volume. Mobility as a qualitative measure was visually assessed, using an arbitrary scale according to recommendations by the [World Health Organization \(1987\)](#); (0–3; 0=no overall motility; 3=high overall motility). The analysis of morphological characteristics included a quantification of headless, angulated, and head-to-head agglutinated spermatozoa (in percent).

Analysis of serum steroid hormone concentrations

Serum hormone concentrations from animals at metestrus and estrus stage of the cycle (assessed daily between 1700 and 1800 h) were determined using the DSL-ACTIVE Progesterone Coated Tube Kit (DSL-5000, Diagnostic Systems Laboratories Inc., Webster, Texas, USA) and the DSL-4400 Estradiol RIA Kit (Diagnostic Systems Laboratories Inc.) with the following modifications to improve E2 delectability. An additional standard value was included (37 pmol/l) to allow reliable detection below the lowest standard value provided by the kit (74 pmol/l). The protocol was also modified with respect to sample incubation (20 h at 7 °C instead of 3 h at 37 °C). The quantification was always conducted in parallel with positive controls with very low (38 pmol/l) and very high (3613 pmol/l) E2 values. Knockout ($n=10$)

and wild-type littermates ($n=9$) were killed after assessment of the estrous cycle stage and blood was drawn from the vena cava. Only animals showing at least three consecutive 4-day estrous cycles were included in the analysis. Adaptation to the procedure of estrous cycle determination was taken as a precaution to minimize stress-related phenomena on the day of serum sample collection.

RT-PCR

Total RNA was isolated from adrenal glands using RNeasy Mini Kit (Qiagen). One microgram total RNA was then reverse transcribed using oligo-dT primer and the Superscript II Reverse Transcriptase kit (Promega). A total of 50 ng cDNA were subsequently amplified by PCR using 1.5U Taq polymerase (Invitrogen Life Technologies), 20 pmol of each primer (Akr1b8-fwd 5'-TgACCAAggCagAATCCTCACC-3' and Akr1b8-rev 5'-AggATgTCaggCaggCTgggC-3'), 0.25 mM dNTPs, 1.5 mM MgCl₂ in a 50 µl reaction volume at 94 °C for 3 min, 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 45 s with 28 cycles. Semi-quantitative conditions were established using increasing amounts of RNA. Products were separated on a 1.2% agarose gel. Expression levels of *Akr1b8* were compared with *Hprt* as a housekeeping control gene (primers sequences: *Hprt*-fwd 5'-gCTggTgAAAAggACCTCT-3' and *Hprt*-rev 5'-CACaggACTAgAACACCTg-3'; 57 °C annealing temperature, reaction conditions as mentioned earlier). Experiments were conducted in three replicates using extracted RNA pooled from adrenal glands of four animals respectively.

Statistical analysis

The significance of differences between groups was assessed using Student's *t*-test and χ^2 -test (for the evaluation of Mendelian ratios). Data are presented as the mean with variation between values indicated as S.E.M. Differences were considered significant when $P<0.05$.

Results

Akr1b7 mRNA is strongly upregulated in theca and stroma cells during proestrus

The induction of *Akr1b7* expression has been shown to occur within 1.5–6 h following hCG administration in immature mice and expression ceases 14 h post-hCG injection (Brockstedt *et al.* 2000). In sexually mature mice, LH is released within the proestrus phase briefly before ovulation occurs, with the highest serum concentrations occurring around 1800 h (Bailey 1987). To investigate whether the observed LH-analog responsiveness occurs *in vivo*, *Akr1b7* mRNA expression in diestrus, proestrus, estrus, and metestrus ovaries was investigated by *in situ* hybridization. High levels of *Akr1b7* mRNA were detected in ovaries obtained from mice in proestrus 6 h post-assumed LH surge (Fig. 1B), while no transcripts were detectable in ovaries during diestrus, estrus, metestrus, and proestrus at the approximate time of the LH surge or 14 h after (Fig. 1A, C and D). *Akr1b7* mRNA expression in proestrus was restricted to theca and interstitial cells of the ovary. In antral follicles, only cells of the theca interna were stained; whereas in preantral follicles, *Akr1b7* transcripts were detected within the entire theca cell layer. In contrast, *Akr1b7* mRNA was never detected in granulosa cells or in

Figure 1 Non-radioactive *in situ* hybridization demonstrates ovarian *Akr1b7* mRNA expression is restricted to the proestrus stage of the estrous cycle. *Akr1b7* expression was analyzed using in ovarian sections from mice in (A) diestrus, (B) proestrus (6 h post-endogenous LH surge), (C) estrus, and (D) metestrus. *Akr1b7* mRNA is only expressed in theca and stromal cells of ovaries during proestrus. CL, corpus luteum; GC, granulosa cells; TC, theca cells; SC, stroma cells; O, oocyte (Original magnification $\times 100$).

corpora lutea (Fig. 1). Thus, the spatial distribution of *Akr1b7* expression in the ovary correlates entirely with our previous data obtained following hCG administration (Brockstedt *et al.* 2000). However, the timeline of *Akr1b7* expression under physiological conditions was more restricted to a period around 6 h post-assumed LH surge and had ceased within 14 h. Furthermore, *Akr1b7* mRNA was not detectable in progesterone-producing corpora lutea of pregnant mice by *in situ* hybridization. No evidence for *Akr1b7* mRNA transcripts, as assessed by northern blot analysis, could be found in the uterus of immature, eCG-treated mice before or after hCG administration or in the placenta from pregnant mice 13 days post-coitum (data not shown).

Targeted disruption of the murine *Akr1b7* gene

The restricted expression of *Akr1b7* in proestrus ovary suggests that this gene product may play an important role in female reproductive physiology, particularly in the ovulatory process. Furthermore, the strong expression level reported in the vas deferens also suggests a role for *Akr1b7* in male reproduction. To investigate the effects on

reproduction *in vivo*, knockout mice were prepared using homologous recombination in ES cells together with Cre-mediated loxP recombination.

Following the targeted insertion of a loxP site 5' of exon 2 and a floxed neomycin selection cassette 3' of exon 4, transient transfection of Cre recombinase in targeted ES cells led to the deletion of exons 2–4 (Fig. 2A), encoding the majority of the amino acids forming the substrate-binding domain (Jez *et al.* 1997, Hyndman *et al.* 2003). The disruption of the *Akr1b7* gene at the genomic level was confirmed by Southern blot analysis (Fig. 2B). The effect of the gene deletion on *Akr1b7* transcripts was assessed using northern blot analysis of *Akr1b7*-expressing organs. An *Akr1b7* cDNA probe detected a truncated transcript in knockout animals, suggesting that splicing from exons 1 to 5 occurs from the transgenic allele (Fig. 2C). No AKR1B7 protein could be detected in knockout mice, confirming the gene deletion (Fig. 2D). Genotyping of the litters of heterozygous intercrosses demonstrated the presence of wild-type, knockout, and heterozygous offspring at the expected Mendelian ratio (females: knockout=50, wild type=51, heterozygous=96, $\chi=0.39$ (df=1), and

Figure 2 Targeted disruption of *Akr1b7*. (A) The targeting vector used to introduce a loxP site (indicated by black arrows) and a floxed selection cassette (PGK-neo) 5' and 3' of exons 2–4 is shown together with the wild-type allele. A negative selection cassette (MC1-tk) used to enrich for homologous recombination was incorporated into the targeting vector. Following transient Cre transfection of homologously recombined ES cell clones, a loxP-mediated deletion occurs, removing exons 2–4 in the knockout allele. Exons appear as numbered boxes and the position of the probe and the BglIII (B) restriction sites are indicated. (B) Southern analysis of BglIII-digested genomic DNA from wild-type (+/+), heterozygote (+/-), and knockout mice (-/-). (C) Northern blot analysis of *Akr1b7* expression in vas deferens, ovary (8 h post-hCG injection), and adrenal gland RNA from wild-type, heterozygote, and knockout mice demonstrates the presence of a truncated transcript in heterozygous and knockout animals. (D) Western analysis of AKR1B7 expression in vas deferens, LH-stimulated ovary, and adrenal gland from wild-type, heterozygous, and knockout mice. Full length AKR1B7 protein is not detectable in knockout animals confirming deletion of targeting domain. Recombinant AKR1B7 (rAKR1B7) was used as a positive control.

$P > 0.05$; males: knockout=53, wild type=49, heterozygous=106, $\chi^2 = 0.89$ (df=1), and $P > 0.05$), indicating that the mutation is not associated with embryonic lethality. Knockout mice appeared phenotypically normal when compared with their heterozygous and wild-type littermates, without obvious detrimental effects on health and viability. All phenotypic observations described were confirmed in *Akr1b7* knockout mice generated from three independent ES cell clones.

***Akr1b7* knockout animals reveal no histological abnormalities in reproductive organs and adrenal gland**

Histological analysis of reproductive organs revealed no abnormalities (Fig. 3A–D). Thus, despite the exceptionally high levels of AKR1B7 protein expression within the vas deferens and the strong LH-dependent regulation in the ovary, apparently normal reproductive tissue histology was observed in the absence of functional AKR1B7 protein. Within the adrenal gland, no obvious morphological differences could be seen between the cortical zona fasciculata in knockout and wild-type littermates

(Fig. 3E and F). Furthermore, comparison of mean organ weights of the adrenal gland of male and female mice, the ovaries, the testes, and the epididymides revealed no apparent alteration in knockout animals when compared with wild-type littermates (Table 1).

Reproductive function in Akr1b7 knockout mice is indistinguishable from wild-type littermates

Despite the lack of apparent histological differences in mice lacking functional *Akr1b7*, the effects of the mutation on reproductive success were investigated. Male and female wild-type and knockout mice were mated with wild-type animals continually throughout their reproductive life span and litter size together with the frequencies of parturitions were ascertained (Table 2). No significant differences ($P > 0.05$) were recorded between wild-type or knockout mice suggesting AKR1B7 protein to be dispensable for reproductive function.

Continuous mating of female mice from sexual maturity onwards results in repeated pregnancies with only few

Figure 3 Histological analysis of AKR1B7-expressing tissues. Photomicrographs of hematoxylin- and eosin-stained sections through the ovary (A, knockout; B, wild type), vas deferens (C, knockout; D, wild type), and adrenal gland (E, knockout; F, wild type). Histology shown is from 20-week-old animals.

Table 1 Organ weights are not significantly altered in *Akr1b7* knockout mice in comparison with wild-type littermate controls.

	Genotype	Adrenal glands (mg)	Testes (mg)	Epididymides (mg)	Ovaries (mg)
Male	Wild-type (<i>n</i> =10)	7.85±0.78	125.7±4.0	48.1±2.4	–
	Knockout (<i>n</i> =8)	7.66±0.79*	126.3±8.2*	49.5±4.3*	–
Female	Wild-type (<i>n</i> =9)	7.77±0.21	–	–	12.22±1.12
	Knockout (<i>n</i> =13)	7.96±0.36*	–	–	12.69±0.82*

Data are presented as the mean ± s.e.m. **P*>0.05.

intermediate estrous cycles. Since *Akr1b7* expression in the ovary is restricted to a distinct period following the LH surge, the effects of the *Akr1b7* gene deletion may be simply masked by the reduced ovulation frequency in permanently mated mice. To test this hypothesis, knockout and wild-type littermates were mated only after they had reached 5 months of age. Housing females in the presence of male pheromones previous to mating thereby ensured maintenance of the estrous cycle. No effect (*P*>0.05) of genotype on the reproductive performance of these mice was observed (Table 2).

Estrous cycle and follicle maturation appears normal in *Akr1b7* knockout mice despite distorted progesterone levels

To further investigate the effects of *Akr1b7* mutation on ovarian function, serum concentrations of progesterone and estradiol were determined by RIA, and the duration of the estrous cycle and follicular maturation were assessed in knockout and wild-type littermates. No significant differences in hormone concentration of estradiol were observed in sera from wild-type and knockout mice during the estrus stage of the cycle (data not shown). However, progesterone levels were found to be significantly elevated (*P*>0.05) in knockout animals (32.5±4.7 nmol/l; *n*=10) during metestrus when compared with wild-type littermates (16.4 nmol/l±3.2; *n*=9) and significantly decreased during estrus in knockout animals (12.9 nmol/l±1.8; *n*=6) when compared with wild-type littermates (18.9 nmol/l±4.6; *n*=6). The estrous cycle stages of mice at 60 days of age were determined daily and monitored over a period of 15 days. No significant differences were found between knockout and wild-type littermates (Fig. 4A). Since ovarian follicles undergo a characteristic maturation process from primordial to antral stages of the leading follicles, the quantitative assessment of follicle maturation might provide a measure of subtle alterations in mice lacking functional AKR1B7 protein. Despite altered serum concentrations of progesterone in estrus (decreased) as well as metestrus (elevated) *Akr1b7* knockout mice exhibit no alterations of the ovarian follicle population in comparison with wild-type littermates, indicating that the mutation of *Akr1b7* does not adversely affect ovarian function (Fig. 4B) and female fertility. The lack of a detoxifying enzyme could impair cell survival and thus be

associated with follicular atresia. However, although our experimental approach limited the assessment to early stages of follicular atresia, a strong atretic phenotype resulting from the targeted deletion would likely influence the overall distribution of non-atretic stages of follicular development; the distributions observed were indistinguishable between knockout and control littermates, thus ruling out this possibility.

***Akr1b7* knockout males reveal normal spermatozoa parameters**

Since male mice are considered to be superfertile, the assessment of functional fertility parameters is a poor reflection of spermatozoa quality and quantity. Subsequently, the potential vas deferens effects of *Akr1b7* mutation on spermatozoa were assessed. Spermatozoa were isolated from the cauda region of the epididymis and the vas deferens of knockout and wild-type mice 22 weeks of age. No significant differences (*P*>0.05) were found in sperm number or quality, as assessed by the proportion of motile spermatozoa as well as their absolute mobility between knockout and wild-type littermates. A slight regression in the percentage of motile sperm was recognized in samples from the vas deferens of knockout animals, which, although not being statistically significant (*P*>0.05), demands further investigation (Table 3). Furthermore, a quantification of morphological abnormalities was conducted to

Table 2 Reproductive success of *Akr1b7* knockout mice at various ages is not impaired in comparison with wild-type littermate controls.

	Age at time of mating	Genotype	Litter size	Litters per month
Male	6 weeks	Wild-type (<i>n</i> =13)	12.8±0.9	0.82±0.13
		Knockout (<i>n</i> =10)	12.3±1.4*	0.88±0.14*
Female	30 days	Wild-type (<i>n</i> =6)	7.11±0.94	1.04±0.10
		Knockout (<i>n</i> =10)	7.00±2.26*	1.02±0.23*
	5 months	Wild-type (<i>n</i> =5)	8.15±2.32	1.07±0.25
		Knockout (<i>n</i> =5)	8.12±1.73*	1.10±0.11*

Data represent the mean litter size (± s.e.m.) and the parturition frequency (litter per month (± s.e.m.)), **P*>0.05.

Figure 4 Estrous cycle duration and follicle maturation in AKR1B7 knockout mice are indistinguishable from wild-type littermates.

(A) Estrous cycle stages were determined daily over a period of 15 days and are cumulatively summarized. Graphs represent the means $\pm$ S.E.M. of knockout ($n=9$; gray bars) and wild-type animals ($n=9$; open bars). No significant differences between mutant and wild-type animals could be observed ($P>0.05$). (B) Bars correspond to the number of follicles per ovary from knockout animals ($n=4$; gray bars) when compared with wild-type littermates ($n=4$; white bars). Classification was assessed according to Pedersen & Peters (1968). Based on apoptotic characteristics, determination of atresia was restricted to stage 5 and antral follicles possessing intact nuclei. No significant differences between mutant and wild-type animals could be observed ($P>0.05$).

determine the percentage of acephalic and head-to-head agglutinated spermatozoa (Table 4). For each parameter, no significant differences between wild-type and knockout animals were observed ($P>0.05$). In single knockout animals, a slightly increased proportion of spermatozoa

with abnormal morphological appearance was observed which, however, lacked statistical significance. Spermatozoa obtained from the non-AKR1B7 expressing cauda epididymis were used as an internal control, for comparisons of spermatozoa collected from the vas deferens and, as expected, no genotype-dependent differences were recorded.

Compensatory effects: Akr1b8 expression levels are not elevated in Akr1b7 knockout animals

AKR1B8 (fibroblast growth factor induced protein-1) is the structurally and functionally closest related protein to AKR1B7 (Seery *et al.* 1998). We therefore investigated whether *Akr1b7*-expressing organs show elevated expression levels of *Akr1b8* mRNA by a compensatory mechanism. Northern blot analysis of ovarian and vas deferens *Akr1b8* transcripts and semi-quantitative RT-PCR of adrenal *Akr1b8* message revealed no apparent induction of *Akr1b8* expression in knockout animals when compared with wild-type littermates (Fig. 5). Specifically, *Akr1b8* is reportedly not expressed in the vas deferens of wild-type mice. In *Akr1b7* knockout mice, no *Akr1b8* mRNA was detected, contradicting a potential compensatory upregulation.

Discussion

Only little is known about the *in vivo* characteristics of AKR1B7 protein and its functional implication into reproductive processes. Within the present study, we first investigated the *in vivo* expression patterns of ovarian *Akr1b7* mRNA during the natural 4-day estrous cycle of mice and demonstrated a rapid induction of *Akr1b7* mRNA following the proestrus stage of the cycle. Confirming our previous results using exogenous LH-analog application, *Akr1b7* mRNA expression is sharply restricted to theca and interstitial cells following the preovulatory LH surge *in vivo*. However, while *Akr1b7* expression following exogenous administration of an LH analog was found to be at a maximum after 14 h, the results of this *in vivo* study revealed the highest expression levels 6 h after the approximate endogenous LH surge, whereas expression had ceased by 14 h. We hypothesize that exogenous hCG administration with doses used in superovulation

Table 3 Spermatozoa parameters for *Akr1b7* knockout mice and wild-type littermate controls^a.

	Cauda epididymis			Vas deferens		
	Sperm count $\times 10^6$ /cauda	Sperm motility (%)	Mobility rank	Sperm count $\times 10^6$ /vas deferens	Sperm motility (%)	Mobility rank
Wild-type ($n=10$)	7.2 ± 1.2	67.8 ± 7.5	2.4 ± 0.2	4.3 ± 1.4	93.2 ± 0.09	2.9 ± 0.03
Knockout ($n=8$)	$4.1 \pm 2.8^*$	$60.3 \pm 12.5^*$	$2.2 \pm 0.37^*$	$7.0 \pm 1.2^*$	$72.8 \pm 10.6^*$	$2.8 \pm 0.15^*$

Data are presented as the mean $\pm$ S.E.M. * $P>0.05$.

^aData were obtained from animals 22 weeks of age. Motility is defined as the percentage of motile spermatozoa. Mobility (the extent or degree of movement) was assessed using an arbitrary scale (0=no mobility to 3=normal mobility).

Table 4 Spermatozoa morphology of *Akr1b7* knockout mice and wild-type littermate controls^a.

	Cauda epididymis			Vas deferens		
	Acephalic spermatozoa (%)	Angulated spermatozoa (%)	Head-to-head agglutination (%)	Acephalic spermatozoa (%)	Angulated spermatozoa (%)	Head-to-head agglutination (%)
Wild-type (n=10)	0.4±0.1	0.4±0.4	1.7±0.1	0.6±0.3	0.3±0.4	1.7±0.9
Knockout (n=8)	1.7±1.2*	2.8±4.5*	4.7±1.4*	1.5±1.1*	1.6±3.4*	5.5±1.9*

Data are presented as the mean ± s.e.m. **P* > 0.05.

^aData were obtained from animals 22 weeks of age.

experiments results in higher serum hormone levels that are maintained for longer periods of time in the circulatory system and result in prolonged mRNA expression.

This distinct expression profile supports the assumption that the AKR1B7 enzyme might play a critical role in the ovulation process, possibly by neutralizing the build-up of toxic intermediates and byproducts of LH-induced steroidogenesis. Following the LH surge, isocaproaldehyde, a preferred substrate of AKR1B7, is produced through a gonadotropin-enhanced P450_{scc} (CYP11A1) activity. Therefore, AKR1B7 might fulfill an isocaproaldehyde detoxifying function not only in the adrenal cortex but also in ovarian theca and stroma cells.

To investigate the potential role of AKR1B7 protein in ovulatory processes, we generated mutant *Akr1b7* mice. Due to the abundant adrenal expression of AKR1B7 which potentially fulfils a vital role in detoxification processes within this organ, a deletion strategy was chosen which would allow maximal flexibility should a lethal phenotype be observed. Using a Cre/loxP approach two types of recombinant clones were obtained for both, the generation of a constitutive knockout lacking AKR1B7 in all tissue types and of a conditional knockout for restricted genetic manipulation of a particular tissue.

Contrary to expectations, constitutive deletion of *Akr1b7* resulted in viable mice, which appeared phenotypically normal in comparison with their wild-type littermates. Furthermore, a lack of statistical variation from the expected Mendelian ratios resulting from heterozygous intercrosses, confirmed that the *Akr1b7* mutation is compatible with normal growth and development, despite the reported embryonic expression of this gene in the actively steroidogenic embryonic adrenal gland (Lau *et al.* 1995). This finding is in contrast to the results of Lefrançois-Martinez who demonstrated that stable transfection of adrenal Y1 cells with *Akr1b7* antisense cDNA led to decreased viability when the cells were exposed to the AKR1B7 substrate isocaproaldehyde and also when increased steroidogenesis was induced within the cells (Lefrançois-Martinez *et al.* 1999). This conflict demonstrates the importance of an *in vivo* approach for functional gene analysis. Since the adult and embryonic adrenal gland is a well-known site of expression of a diverse array of AKR family members (Maser & Bannenberg 1994, Lau *et al.* 1995), the potential detoxifying role of AKR1B7 may be replaced in the mutant

mouse by a related enzyme. Potentially, these compensatory mechanisms are not preserved in the aforementioned *in vitro* models. Despite the lack of a demonstrable reproductive phenotype in this work, future studies will investigate the effects of *Akr1b7* deletion following physiological challenge, conditions which are potentially associated with an increased requirement for detoxifying AKR1B7 enzyme.

As shown by the determination of diverse parameters of female reproductive function, such as litter size, parturition frequency, estrous progression, and follicle staging analysis, AKR1B7 expression is dispensable for ovarian function. Furthermore, recurrent ovulations without intermitting pregnancies, and thus, the long-term effects of the gene disruption did not have an impact on female fertility and no alterations in female reproductive lifespan were observed. Alterations found in serum hormone concentrations of progesterone in metestrus- and estrus-stage knockout females seemingly failed to affect reproductive performance. Progesterone is mainly synthesized following rupture of the Graafian follicle in the corpus luteum. However, other tissues producing progesterone include the placenta, theca cells of early follicle stages, and the adrenal gland. Since AKR1B7 protein expression is restricted to theca cells shortly before ovulation (Brockstedt *et al.* 2000) and to the zona fasciculata of the adrenal gland (Lau *et al.* 1995,

Figure 5 *Akr1b8* mRNA expression analysis of *Akr1b7* knockout animals. Ovarian and vas deferens tissue was examined by northern blot analysis (A). No induction of mRNA expression in knockout animals when compared with wild-type littermates was observed. Importantly, no *Akr1b8* mRNA was detected in the vas deferens of knockout males. The 28S and 18S rRNAs were used as loading controls. Adrenal *Akr1b8* expression was investigated by semi-quantitative RT-PCR with no apparent differences in *Akr1b8* expression between knockout and wild-type animals (B). Expression of *Hprt* was used as a loading control.

Aigueperse *et al.* 1999), effects of the targeted deletion of *Akr1b7* in the adrenal gland could possibly interfere with hormonal feedback mechanisms affecting hormone levels depending on the estrous cycle stage of *Akr1b7* knockout animals. Furthermore, malfunction of the adrenal gland could have effects on metabolic processes rather than reproductive performance. These hypotheses deserve further investigation.

A gross assessment of spermatozoa collected from the vas deferens of male knockout mice similarly confirmed that male reproductive function is also independent of AKR1B7 protein expression within this organ. However, a trend towards decreased motility in knockout mice was observed and might indicate subtle differences missed due to the arbitrary scale analysis used in the present study. This deserves a more detailed investigation. However, a degree of variation in fertility parameters (including sperm motility) has been reported between different mouse strains (Silver 1995) and the observed motility difference may simply be due to the mixed genetic background used in this study, resulting from the use of 129 strain ES cells. The establishment of this mutation on different purebred genetic backgrounds would help the quantification of a potential reproductive phenotype. Indeed, the use of a sub-fertile strain such as 129 could perhaps reveal a fertility phenotype as yet masked by the C57BL/6 strain used in this study.

The lack of an obvious impairment of spermatozoa is surprising while considering the high proportion of AKR1B7 protein within the soluble protein fraction of vas deferens. It has, however, been shown that this high level of AKR1B7 expression is unique for the vas deferens of the mouse when compared with other rodent species (Taragnat *et al.* 1990, Schnolzer *et al.* 1992, Aigueperse *et al.* 1999). The murine expression has been shown to be the result of a 77 bp LINE element-derived sequence insertion, which is specific to the murine AKR1B7 promoter and conveys androgen responsiveness. This observation has led to a discussion concerning the selective advantage of a high AKR1B7 expression level in the murine vas deferens (Val *et al.* 2004). One explanation suggested an involvement of AKR1B7 in prostaglandin synthesis, which has been shown to be comparatively high within the murine vas deferens (Badr 1975, Marshburn *et al.* 1989). The results of this study, however, suggest that functional AKR1B7 protein is dispensable for normal reproductive function of the murine vas deferens, indicating that any selective advantage is subtle.

The observed lack of a reproductive phenotype in both male and female knockout mice, along with the presence of other AKRs within reproductive tissues, suggested that compensatory mechanisms might be at play. We therefore investigated the possible induction of *Akr1b8* expression – the structurally and functionally closest related protein (Seery *et al.* 1998). Significantly, we found no indication for an expression of *Akr1b8* in the vas deferens of neither knockout nor wild-type

animals. While, in contrast to *Akr1b7*, *Akr1b8* expression in the ovary is independent from LH, in the adrenal gland and in the post-LH ovary *Akr1b7* and *Akr1b8* are expressed at similarly high levels. The lack of an observed strong upregulation of *Akr1b8* in these tissues argues against the presence of a compensatory mechanism involving this related family member.

Since fertility serves the purpose of species maintenance, the selective pressure for such mechanisms within reproductive physiology is high. Therefore, several distinct defense systems presumably act together and might compensate for a defective mechanism. Other members of the AKR gene family, besides *Akr1b8*, as well as enzymes with similar properties, e.g., glutathione-S-transferases, are likely candidates which could replace some of the functions of AKR1B7.

In addition to the potential role in detoxification of isocaproaldehyde resulting from steroidogenesis, a role for this AKR in oxidative stress is suggested by its substrate specificity for 4-HNE. This toxic intermediary is released following lipid peroxidation and is considered to be a central mediator of the toxic effects resulting from oxidative stress. Besides the cytotoxic characteristics of 4-HNE endangering cell viability and causing pathological alterations, for example, in disorders such as atherosclerosis (Palinski *et al.* 1989, Rosenfeld & Ross 1990) and neurodegenerative diseases (Yoritaka *et al.* 1996, Ando *et al.* 1998, Calingasan *et al.* 1999), this compound has also been shown to induce a variety of cellular response processes, e.g., apoptosis, cell growth, detoxification, and protein turnover (Uchida 2003 and references therein). Furthermore, when compared with free radicals, 4-HNE is relatively stable and through diffusion can act upon target cells far away from the original source of the lipid peroxidation (Dianzani *et al.* 1999). Therefore, a potential physiological role for AKR1B7 in the control of 4-HNE levels may have effects in a far more subtle manner than expected, leading to diverse effects on cellular physiology. Recently, an association of AKR1B7 protein with the intestinal nuclear receptor liver X receptor (LXR), acting as sterol sensor (Repa & Mangelsdorf 2002, Volle *et al.* 2004), has been reported. These interactions and the multiple functions of adrenal glucocorticoids in inflammatory, energy, and stress reactions are starting points for future experiments which may reveal a potential role of AKR1B7 protein in non-reproductive tissues.

Acknowledgements

This work was supported by Bayer Schering Pharma AG, Berlin, Germany. The authors wish to thank Dagmar Sonnenberg for her excellent technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Aigueperse C, Martinez A, Lefrancois-Martinez A, Veyssiere G & Jean C 1999 Cyclic AMP regulates expression of the gene coding for a mouse vas deferens protein related to the aldo-keto reductase superfamily in human and murine adrenocortical cells. *Journal of Endocrinology* **160** 147–154.
- Aitken R 1994 A free radical theory of male infertility. *Reproduction, Fertility, and Development* **6** 19–23.
- Ando Y, Brännström T, Uchida K, Nyhlin N, Näsman B, Suhr O, Yamashita T, Olsson T, Salhy ME, Uchino M *et al.* 1998 Histochemical detection of 4-hydroxynonenal protein in Alzheimer amyloid. *Journal of the Neurological Sciences* **156** 172–176.
- Badr F 1975 Prostaglandin levels in tissues of the male reproductive system in six strains of mice. *Endocrinology* **96** 540–543.
- Bailey K 1987 Diurnal progesterone rhythms in the female mouse. *Journal of Endocrinology* **112** 15–21.
- Bohren K, Bullock B, Wermuth B & Gabbay K 1989 The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. *Journal of Biological Chemistry* **264** 9547–9551.
- Brockstedt E, Peters-Kottig M, Badock V, Hegele-Hartung C & Lessl M 2000 Luteinizing hormone induces mouse vas deferens protein expression in the murine ovary. *Endocrinology* **141** 2574–2581.
- Burstein S & Gut M 1971 Biosynthesis of pregnenolone. *Recent Progress in Hormone Research* **27** 303–349.
- Butcher RL, Collins WE & Fugo NW 1974 Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17 β throughout the 4-day estrous cycle of the rat. *Endocrinology* **94** 1704–1708.
- Calingasan NY, Uchida K & Gibson GE 1999 A novel marker of oxidative stress in Alzheimer's disease. *Journal of Neurochemistry* **72** 751–756.
- Comporti M 1998 Lipid peroxidation and biogenic aldehydes: from the identification of 4-hydroxynonenal to further achievements in biopathology. *Free Radical Research* **6** 623–635.
- Constantopoulos G, Carpenter A, Satoh P & Tchen T 1966 Formation of isocaproaldehyde in the enzymatic cleavage of cholesterol side chain by adrenal extract. *Biochemistry* **5** 1650–1652.
- Davies B, Baumann C, Kirchhoff C, Ivell R, Nubbemeyer R, Habenicht UF, Theuring F & Gottwald U 2004 Targeted deletion of the epididymal receptor HE6 results in fluid dysregulation and male infertility. *Molecular and Cellular Biology* **24** 8642–8648.
- Dianzani M, Barrera G & Parola M 1999 4-Hydroxy-2,3-nonenal as a signal for cell function and differentiation. *Acta Biochimica Polonica* **46** 61–75.
- Esterbauer H, Schaur R & Zollner H 1991 Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine* **11** 81–128.
- Goldring N, Durica JM, Lifka J, Hedin L, Ratoosh SL, Miller WL, Orly J & Richards JS 1987 Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. *Endocrinology* **120** 1942–1950.
- Hegele-Hartung C, Siebel P, Peters O, Kosemund D, Muller G, Hillisch A, Walter A, Kraetzschmar J & Fritzemeier KH 2004 Impact of isotype-selective estrogen receptor agonists on ovarian function. *PNAS* **101** 5129–5134.
- Hyndman D, Bauman D, Heredia V & Penning TM 2003 The aldo-keto reductase superfamily homepage. *Chemico-Biological Interactions* **143–144** 621–631.
- Jez J, Flynn T & Penning T 1997 A new nomenclature for the aldo-keto reductase superfamily. *Biochemical Pharmacology* **54** 639–647.
- Laemmli U 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Lau ET, Cao D, Lin CS, Chung S & Chung S 1995 Tissue-specific expression of two aldose reductase-like genes in mice: abundant expression of mouse vas deferens protein and fibroblast growth factor-regulated protein in the adrenal gland. *Biochemical Journal* **312** 609–615.
- Lefrancois-Martinez A-M, Tournaire C, Martinez A, Berger M, Daoudal S, Tritsch D, Veyssiere G & Jean C 1999 Product of side-chain cleavage of cholesterol, isocaproaldehyde, is an endogenous specific substrate of mouse vas deferens protein, an aldose reductase-like protein in adrenocortical cells. *Journal of Biological Chemistry* **274** 32875–32880.
- Marshburn P, Clark M & Shabanowitz R 1989 Immunohistochemical localization of prostaglandin H synthase in the epididymis and vas deferens of the mouse. *Biology of Reproduction* **41** 491–497.
- Martinez A, Aigueperse C, Val P, Dussault M, Tournaire C, Berger M, Veyssiere G, Jean C & Lefrancois Martinez A 2001 Physiological functions and hormonal regulation of mouse vas deferens protein (AKR1B7) in steroidogenic tissues. *Chemico-Biological Interactions* **130–132** 903–917.
- Maser E & Bannenberg G 1994 11-beta-Hydroxysteroid dehydrogenase mediates reductive metabolism of xenobiotic carbonyl compounds. *Biochemical Pharmacology* **47** 1805–1812.
- Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D & Witztum JL 1989 Low density lipoprotein undergoes oxidative modification *in vivo*. *PNAS* **86** 1372–1376.
- Pedersen T & Peters H 1968 Proposal for a classification of oocytes and follicles in the mouse ovary. *Journal of Reproduction and Fertility* **17** 555–557.
- Pizzimenti S, Laurora S, Briatore F, Ferretti C, Dianzani M & Barrera G 2002 Synergistic effect of 4-hydroxynonenal and PPAR ligands in controlling human leukemic cell growth and differentiation. *Free Radical Biology and Medicine* **32** 233–245.
- Repa JJ & Mangelsdorf DJ 2002 The liver X receptor gene team: potential new players in atherosclerosis. *Nature Medicine* **8** 1243–1248.
- Richards J, Hedin L, Lifka J, Ratoosh S, Durica J & Goldring NB 1987 Ovarian follicular development: from physiology to molecular biology. *Recent Progress in Hormone Research* **43** 231–276.
- Rosenfeld M & Ross R 1990 Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis* **10** 680–687.
- Rugh R 1968 In *The Mouse, Its Reproduction and Development*, pp 38–39. Minneapolis: Burgess Publishing Company.
- Schnolzer M, Alewood P, Jones A, Alewood D & Kent S 1992 *In situ* neutralization in boc-chemistry solid phase peptide synthesis. rapid, high yield assembly of difficult sequences. *International Journal of Peptide and Protein Research* **40** 180–193.
- Seery L, Nestor P & FitzGerald G 1998 Molecular evolution of the aldo-keto reductase gene superfamily. *Journal of Molecular Evolution* **46** 139–146.
- Silver L 1995 *Mouse Genetics. Concepts and Applications*, New York: Oxford University Press.
- Smith A 1991 Culture and differentiation of embryonic stem cells. *Journal of Tissue Culture Methods* **13** 89–94.
- Taniguchi H & Hayashi N 1998 A liquid chromatography/electrospray mass spectrometric study on the post-transcriptional modification of tRNA. *Nucleic Acids Research* **26** 1481–1486.
- Taragnat C, Berger M & Jean C 1986 Identification and androgen-dependence of proteins in the mouse vas deferens. *International Journal of Andrology* **9** 299–311.
- Taragnat C, Berger M & Jean C 1988 Preliminary characterization, androgen-dependence and ontogeny of an abundant protein from mouse vas deferens. *Journal of Reproduction and Fertility* **83** 835–842.
- Taragnat C, Berger M & Jean C 1990 Tissue and species specificity of mouse ductus deferens protein. *Journal of Andrology* **11** 279–286.
- Uchida K 2003 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in Lipid Research* **42** 318–343.
- Val P, Aigueperse C, Ragazzon B, Veyssiere G, Lefrancois-Martinez A-M & Martinez A 2004 Adrenocorticotropin/3',5'-cyclic AMP-mediated

- transcription of the scavenger *akr1-b7* Gene in adrenocortical cells is dependent on three functionally distinct steroidogenic factor-1-responsive elements. *Endocrinology* **145** 508–518.
- Volle DH, Repa JJ, Mazur A, Cummins CL, Val P, Henry-Berger J, Caira F, Veyssiere G, Mangelsdorf DJ & Lobaccaro J-MA** 2004 Regulation of the aldo-keto reductase gene *akr1b7* by the nuclear oxysterol receptor LXR α (liver X receptor- α) in the mouse intestine: putative role of LXRs in lipid detoxification processes. *Molecular Endocrinology* **18** 888–898.
- World Health Organization** 1987 *World Health Organization Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*, Cambridge, UK: Cambridge University Press.
- Yang X, Li C, Xu X & Deng C** 1998 The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *PNAS* **95** 3667–3672.
- Yi P, Zhan D, Samokyszyn V, Doerge D & Fu P** 1997 Synthesis and ³²P-postlabeling/high-performance liquid chromatography separation of diastereomeric 1,N2-(1,3-propano)-2'-deoxyguanosine 3'-phosphate adducts formed from 4-hydroxy-2-nonenal. *Chemical Research in Toxicology* **11** 1259–1265.
- Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman E & Mizuno Y** 1996 Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *PNAS* **93** 2696–2701.

Received 13 January 2007

First decision 6 February 2007

Accepted 10 April 2007