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.

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, prosta-glandins, 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 prokaryotes, eu-bacteria, fungi, and archaeabacteria 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 (adrenocorticotropic hormone (ACTH); Lefrancois-Martinex 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; Lefrancois-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<sup>tm1.1Fth</sup>/Akr1b7<sup>tm1.1Fth</sup>) 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 Dral 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′-gAAgCTTAagCCagCATTATCACCTgTgTCag-3′ and 5′-TAagCTTAgtCAATgATTTgAACTCTGCTATg -3′.
Generation of knockout mice

Type III recombinant 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’-TgTCAAACTCCTCTCCGATA TACCT-3’) and two reverse primers, one hybridizing to the floxed region, directly upstream of exon 2 and the other hybridizing downstream of exon 2 (5’-TTgGATAA-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<sup>tm1.1Fth</sup> (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 (ALLPKDNKGKVLLSKSTFLDA) 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.

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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 layers 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., Webster, Texas, USA) 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’-TGACCAAGGCAGATCCTACC-3’ and Akr1b8-rev 5’-AggATgTCAGgCAGgCTgggC-3’), 0.25 mM dNTPs, 1.5 mM MgCl\(_2\) in a 50 \(\mu\)l 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’-gCTggTgAAAACgACCTCT-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 \(\chi^2\)-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 granulosa cells.
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, χ = 0.39 (df = 1), and...
$P > 0.05$; 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](https://www.reproduction-online.org) 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.
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 decreased (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 litter mates, 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.

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

Data represent the mean litter size (± S.E.M.) and the parturition frequency (litter per month (± S.E.M.)), *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 knock-out 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 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.

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<thead>
<tr>
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<th>Cauda epididymis</th>
<th>Vas deferens</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sperm count×10⁷/cauda</td>
<td>Sperm motility (%)</td>
</tr>
<tr>
<td>Wild-type (n=10)</td>
<td>7.2±1.2</td>
<td>67.8±7.5</td>
</tr>
<tr>
<td>Knockout (n=8)</td>
<td>4.1±2.8*</td>
<td>60.3±12.5*</td>
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Data are presented as the mean±S.E.M., *P<0.05.

*Data 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).
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 P450scc (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 fulfills 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 Lefrancois-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 (Lefrancois-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, estrus 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 (Brocksstedt et al. 2000) and to the zona fasciculata of the adrenal gland (Lau et al. 1995, etc.)
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 C57BL6 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, Scholzer 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.

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