

Aard is specifically up-regulated in Sertoli cells during mouse testis differentiation

TERJE SVINGEN, ANNEMIEK BEVERDAM, PALI VERMA, DAGMAR WILHELM and PETER KOOPMAN*

Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, Australia

ABSTRACT *Aard* (alanine and arginine rich domain) is a gene of unknown function, previously reported to show sexually dimorphic expression in fetal mouse gonads. Here we describe the spatio-temporal expression profile of *Aard* during gonad development. The period of elevated mRNA expression coincides with early differentiation of the testis and is limited to Sertoli cells of the developing testis cords. Although low levels of *Aard* transcripts were detected in other tissues by quantitative RT-PCR assays, high levels of *Aard* expression is specific to the testis in both embryonic and adult mice.

KEY WORDS: *Aard*, gonad, sex differentiation, testis cord, Sertoli cell

Aard (alanine and arginine rich domain) was initially cloned from and characterized in, postnatal rat lung tissue and named *rA5D3* (GenBank access. no. NM_145093). Using differential display assays to screen for differentially expressed genes during lung development, Blomberg *et al.* (2002) found *Aard* transcripts to be up-regulated from 2 days *post partum* (dpp), peaking at 8 dpp. *Aard* was also shown to be expressed in kidney, heart and testis in the early postnatal rat. However, the expression levels in testis were significantly higher than in other tissues in the period between 2 and 14 dpp. By combining Northern analyses, library screening and RNase protection assays, Blomberg *et al.* (2002) further report the potential existence of a three-amino acid truncated isoform of AARD. The function of AARD protein in any tissue is yet to be determined.

The mouse *Aard* homologue (GenBank access. no. NM_175503) was first identified as a gene showing sexually dimorphic expression in fetal gonads by PCR-based cDNA subtraction analyses and confirmed to be restricted to the sex cords of XY gonads by whole-mount *in situ* hybridization (Menke and Page, 2002). Male-specific expression of *Aard* in developing gonads has been confirmed in separate studies (Menke *et al.*, 2003; Bouma *et al.*, 2004; Nef *et al.*, 2005; Beverdam and Koopman, 2006). However the temporal and cell-specific expression profile of *Aard* in the developing mouse fetus is not yet known. We therefore analysed the expression pattern of *Aard* in mouse embryos with particular emphasis on gonadal tissues.

Aard expression is significantly up-regulated in the gonads of male fetal mice

Utilising quantitative real-time RT-PCR (qRT-PCR) protocols, *Aard* expression was examined in several tissues of 13.5 days *post*

coitum (dpc) mouse embryos (Fig. 1A). *Aard* transcripts were detectable at low levels in all tissues examined including brain, liver, lung, limb, heart, kidney and ovary. However, in the testis, *Aard* was significantly up-regulated. These results were confirmed by section *in situ* hybridization (SISH) on 13.5 dpc male embryos (Fig. 1B). A strong hybridization signal was detected within the testis, with only a weak signal detected in other tissues.

Aard is specifically up-regulated in XY gonads during sex differentiation

Further experiments were undertaken to determine whether *Aard* expression was up-regulated before or after sex determination. qRT-PCR and whole-mount *in situ* hybridization (WISH) analyses showed a clear up-regulation of *Aard* transcription in XY gonads compared to XX gonads (Fig. 2). By qRT-PCR assays, *Aard* was detected in the XY gonads from 11.5 dpc, but with a significant increase in mRNA levels thereafter, peaking at around 12.5 dpc (Fig. 2A). WISH experiments confirmed this expression profile, with robust levels of *Aard* expression observed from 12.5 dpc, with hybridisation signals limited to the cord structures of the developing testis (Fig. 2B). Sex determination is initiated around 10.5 dpc through the function of the Y-linked gene *Sry* (Koopman *et al.*, 1990) and testis cords differentiate between 12 and 13.5 dpc.

Abbreviations used in this paper: Aard, alanine and arginine rich domain; Dig, digoxigenin; dpc, days post coitum; dpp, days post partum; PBS, phosphate-buffered saline; PFA, paraformaldehyde; qRT-PCR, quantitative real-time; RT-PCR, reverse transcription polymerase chain reaction; SISH, section *in situ* hybridization; WISH, whole-mount *in situ* hybridization;

*Address correspondence to: Peter Koopman, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia.
Fax: +61-7-3346-2101. e-mail: p.koopman@imb.uq.edu.au

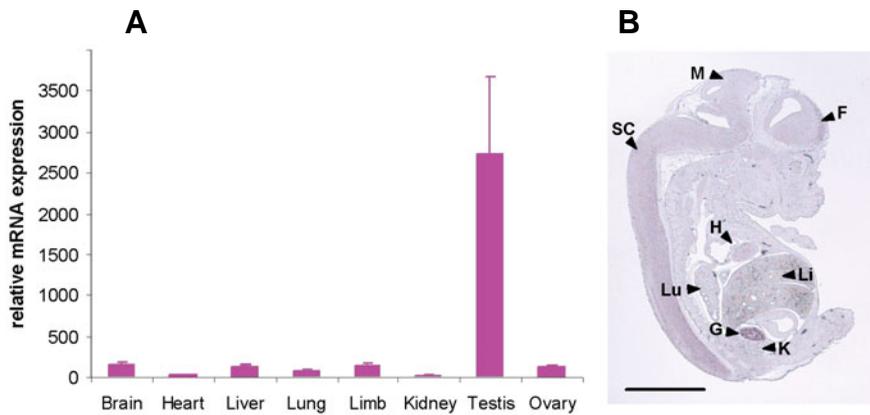


Fig. 1. Tissue-specific expression of *Aard* in the mouse embryo. (A) qRT-PCR analysis of *Aard* mRNA expression relative to 18S RNA (Mean \pm SEM of three independent experiments, each performed in triplicate) in several 13.5 dpc mouse embryonic tissues. For each experiment, non-gonadal tissues were pooled from at least three littermate embryos; testes and ovaries were pooled from at least six littermates. The testis expresses *Aard* at a significantly higher level than other embryonic tissues, including ovary. (B) DIG-labelled sagittal SISH of a 13.5 dpc male embryo. Strong *Aard* expression is evident within the gonad compared to any other tissue. M, midbrain; F, forebrain; SC, spinal cord; H, heart; Lu, lung; Li, liver; G, gonad; K, kidney. Scale bar, 2 mm.

Our data therefore suggest that elevated levels of *Aard* expression are associated with the most active phase of testis cord differentiation.

Aard localizes to Sertoli cells in the XY gonads

WISH with busulfan-treated fetal gonads and RT-PCR on *W^f/W^e* gonads has previously shown that *Aard* expression persists in germ cell-depleted XY gonads; however these experiments could not exclude the possibility that *Aard* might be expressed within the germ cells in addition to somatic cells (Menke and Page, 2002). To clarify this issue, we first repeated WISH and qRT-PCR analyses on 13.5 dpc gonads of *W^f/W^e* mice (Fig. 3), which are devoid of germ cells (Buehr *et al.*, 1993). As previously shown, *Aard* transcripts are detectable by WISH within the cord structures of germ cell-depleted gonads. Further, qRT-PCR data showed persistent *Aard* expression in the *W^f/W^e* gonads, as did the Sertoli cell marker *Sox9*, whereas the germ cell marker *Oct4* was undetected in the *W^f/W^e* gonads. Hence, *Aard* expression is not

dependent on the presence of germ cells.

To further define the cell type expressing *Aard* in the testis cords, we performed SISH using gene specific probes for *Aard* and the cell-specific markers *Amh* (Sertoli cells) and *Oct4* (germ cells). *Aard* hybridization signal was detected in the Sertoli cells (Fig. 4 B,E), as was *Amh* (Fig. 4A,D), but not in the germ cells identified by *Oct4* hybridization (Fig. 4C,F). Sertoli cells support the germ cells in the differentiated male gonad, with their cytoplasmic processes extending between the germ cells. As shown in Fig. 4, *Aard* staining is most prominent in the stellate cytoplasm of the Sertoli cells, but is absent from the annular cytoplasm that characterize the germ cells (compare Fig. 4 E,F). No staining was detected within any other cell types of the testis, including peritubular myoid cells. Hence, *Aard* expression localizes to the Sertoli cells in the XY gonad.

Aard expression remains testis-specific in adult mice

Finally, the expression of *Aard* was examined in adult mouse

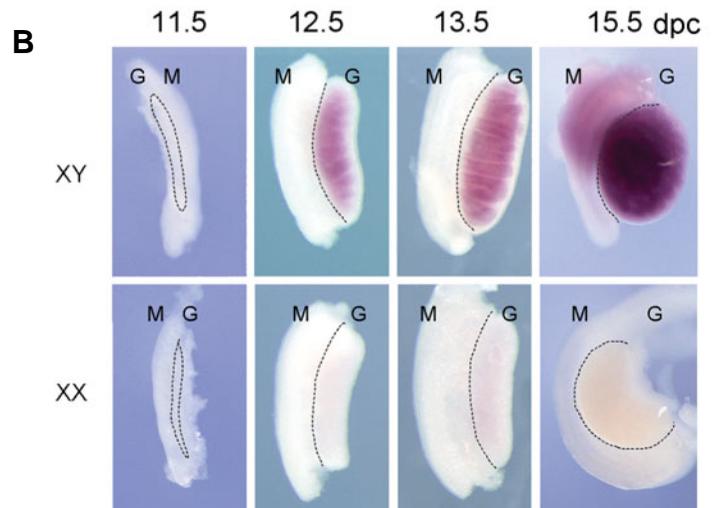
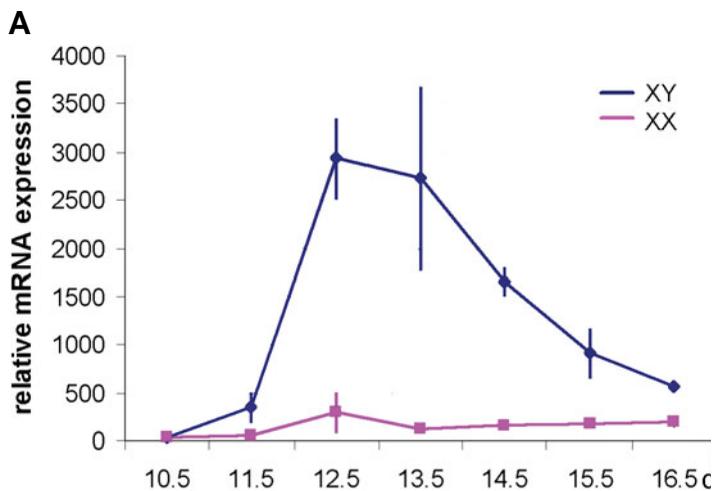
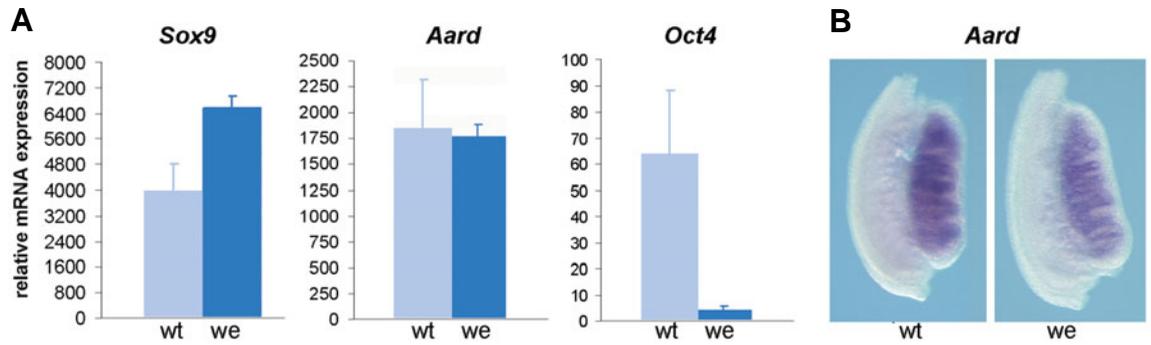


Fig. 2. Temporal expression analysis of *Aard* in the fetal gonads. (A) qRT-PCR analysis of *Aard* mRNA expression relative to 18S RNA (Mean \pm SEM of three independent experiments, each performed in triplicate) showing male-specific up-regulation in fetal gonads. Individual experiments were performed on RNA obtained from pooled gonads from at least six littermates. Stage 10.5 dpc and 11.5 dpc gonads had mesonephroi attached, whereas later-stage gonadal samples had mesonephroi removed. *Aard* transcripts are detected in male gonads from 11.5 dpc, with transcription levels increasing markedly and peaking at 12.5 dpc, after which mRNA levels decreases steadily towards 16.5 dpc. (B) WISH analysis confirming male-specific up-regulation of *Aard* in fetal gonads between 11.5 and 12.5 dpc. Apparent strong signal in XY 15.5 dpc gonad is due to sample thickness compared to early-stage gonads. Abbreviations: M, mesonephros; G, gonad.

Fig. 3. Germ cell-independent Aard expression in 13.5 dpc testes.

(A) qRT-PCR analysis of total RNA extracted from W^e/W^e versus pooled wild-type (+/+) and heterozygous (+/ W^e) littermate gonads using gene specific primers for Aard, Sox9 (Sertoli cell marker) and Oct4 (germ cell marker) relative to 18S RNA (mean \pm SEM of three independent experiments).

Individual experiments were performed in triplicate on RNA obtained from pooled gonads from 3-4 littermates. Both Aard and Sox9 expression are maintained in germ cell-depleted gonads, whereas Oct4 expression was lost in the W^e/W^e samples. **(B)** WISH analysis comparing Aard gene expression in W^e/W^e and wild-type (+/+) male gonads. Aard-specific staining is seen in wild-type and littermate W^e/W^e gonads. (wt, wild type [+ / +] and heterozygous [+/ W^e] littermates; we, homozygous W^e/W^e mutants).



tissues of both sexes. Quantitative analyses showed *Aard* to be testis-specifically up-regulated in postnatal mice, with only relatively low levels of expression detected in other tissues such as ovary, lung, limb and kidney (Fig. 5). These findings suggest a further role for *Aard* in postnatal testis function and/or maintenance of testis cell lineages.

In summary, we have shown that *Aard* is specifically up-regulated in testes of the developing mouse embryo relative to ovaries and other tissues. Moreover, *Aard* mRNA is specifically expressed in Sertoli cells of XY gonads during sex differentiation, with transcription levels peaking around 12.5 dpc when histological differentiation of the testis is most active. Strong testis-specific expression of *Aard* was also detected in the adult mouse. The presence of a predicted leucine-zipper domain and a phosphorylation site in the AARD protein sequence suggest that AARD may be involved in transcriptional regulation or intracellular signal transduction pathways (Blomberg *et al.*, 2002). Hence, our data suggest that AARD may be involved in the regulatory hierarchy

governing testis cord formation and potentially maintenance of the Sertoli cell lineage in the postnatal testis.

Experimental Procedures

Animals

Embryos were collected from timed matings of the CD1 outbred strain and the $c\text{-Kit}^{W^e/+}$ inbred strain, with noon of the day on which the mating plug was observed designated 0.5 dpc. Embryos 11.5 dpc or younger were sexed by *Zfy* RT-PCR. Primers were *Zfy.F*: 5'-CCTATTGCATGGACTGCAGCTTATG and *Zfy.R*: 5'-GACTAGACATGTCTTAACATCTGTCC. Later-staged embryos were sexed by morphological assessment of gonads.

RNA isolation, cDNA synthesis and SYBR-green qRT-PCR

Fetal gonads were dissected and pooled according to sex and developmental stage (one litter per pool) then total RNA isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. cDNA was synthesised from 1 μ g total RNA by reverse

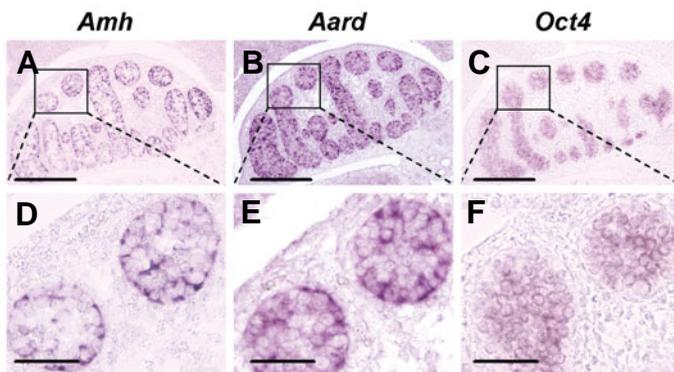
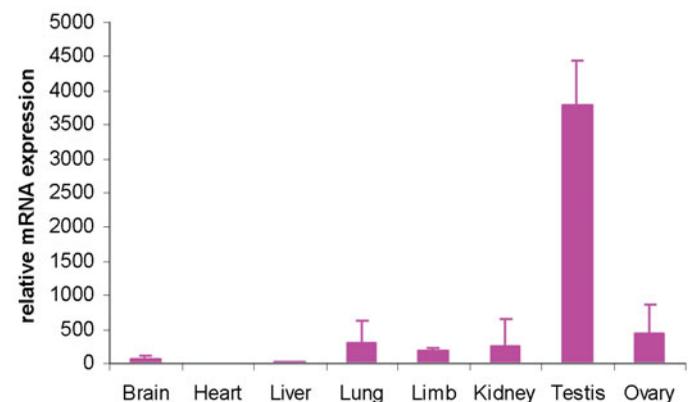


Fig. 4. Cell-specific Aard expression in the fetal male gonad (Left). SISH staining of 13.5 dpc male gonads using specific probes for Amh (A,D), Aard (B,E) and Oct4 (C,F). Aard is expressed in the testis cords and localizes to Sertoli cells. Aard is not expressed in germ cells, with staining different to that observed for the germ cell marker Oct4. Boxed areas indicate regions shown in the square below. Scale bars: 200 μ m (A,B,C); 100 μ m (D,E,F).

Fig. 5. Testis-specific expression of Aard in the adult mouse (Right). qRT-PCR analysis of Aard mRNA expression relative to 18S RNA (Mean \pm SEM of three independent experiments, each performed in triplicate) in adult mouse tissues. The three independent biological samples were obtained from one male and two female adult mice, with the exception of gonadal tissues, which are represented by three individual ovaries and testes. Aard shows high expression in testis compared to any other tissues examined.



transcription (Superscript III, Invitrogen) using random primers (Promega) according to manufacturer's instructions (Invitrogen).

qRT-PCR experiments were performed in triplicate and repeated using three different biological samples and comprised of 25 µl reactions containing SYBR green PCR master-mix (Applied Biosystems), 3.75 µM each forward and reverse primers and 1 µl of cDNA, prepared as described using an ABIPrism-7000 Sequence Detector System. PCR was conducted over 40 cycles of 95°C for 15 s and 60°C for 1 min in a two-step thermal cycle, preceded by an initial 10 min step at 95°C to activate the Amplitaq Gold DNA polymerase. Primers used were:

rtAard.F: 5'-GAAGGGAGGAGGGGTGAG and

rtAard.R: 5'-GGCAAACCTTTAGTGCTTTGGT

rtSox9.F: 5'-AGTACCCGCATCTGCACAAC and

rtSox9.R: 5'-TACTTGTAAATCGGGGTGGTCT

rtOct4.F: 5'-TGCGGAGGGATGGCATACTG and

rtOct4.R: 5'-GCACAGGGCTCAGAGGAGGTTTC. To adjust for variation in input cDNA, samples were normalized against 18S rRNA using the ΔCt method. 18S primers used were

rt18S.F: 5'-GATCCATTGGAGGGCAAGTCT and

rt18S.R: 5'-CCAAGATCCAACACTACGAGCTTTTT. Dissociation curve analyses were performed. The use of 18S rRNA as the normalizing gene was further validated by repeating the analyses relative to the housekeeping gene *TFIIID*, which gave the same results as using 18S (Data not shown).

Digoxigenin whole-mount and section in situ hybridization

Embryos and dissected gonads/mesonephroi were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for several hours at 4°C. WISH with DIG-labelled RNA probes were carried out essentially as described by (Hargrave *et al.*, 2006). For SISH samples, whole embryos were processed and mounted in paraffin wax and stored at 4°C. SISH was performed on 7 µm sagittal sections that were dewaxed, rehydrated and incubated in 5 µM/ml *proteinase K* for 20 min at room temperature (rt), after which samples were washed in PBS. Sections were re-fixed with 4% PFA for 10 min at rt, acetylated and pre-hybridized with hybridization solution (50% formamide, 5X SSC, 5X Denhardt's, 250 µg/ml yeast RNA, 500 µg/ml herring sperm DNA) for 2 h at rt. Hybridization (0.5 µg/ml probe in hybridization solution) was performed overnight at 60°C. Slides were washed in 5X SSC for 5 min, 0.2X SSC for 1 h at 60°C, 0.2X SSC for 5 min at rt and NT buffer (150 mM NaCl, 50 mM Tris-HCl pH7.5) for 5 min at rt, before blocking for 2 h with blocking solution (10% heat-inactivated sheep serum in NT buffer) in a humidified chamber. Anti-DIG antibody (Roche) in blocking solution (1:2000) was added to the slides and incubated overnight at 4°C. Unbound antibodies were removed by washing three times in NT buffer. Section were equilibrated in NTM buffer (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl₂) and incubated in colour solution (3.5 µl BCIP (Roche), 3.5 µl NBT (Roche) per ml NTM buffer) until purple staining was satisfactory.

A 233 bp *Aard* fragment was cloned from position 638-871 (GenBank access. no. NM_175503) into pGEM T-Easy vector (Promega) and targets the 3' UTR, thus detecting both isoforms reported by Blomberg *et al.* (2002). Primers used were Aard.F: 5'-GCTCTGAAACCCTCCTACC

and Aard.R: 5'-TGCTTTGGTTGTCTTCCACC. The probes for *Amh* and *Oct4* were made as previously described (Münsterberg and Lovell-Badge, 1991; Schepers *et al.*, 2003).

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