**ARX/Arx is expressed in germ cells during spermatogenesis in both marsupial and mouse**

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Abstract

The X-linked aristaless gene, ARX, is essential for the development of the gonads, forebrain, olfactory bulb, pancreas, and skeletal muscle in mice and humans. Mutations cause neurological diseases, often accompanied by ambiguous genitalia. There are a disproportionately high number of testis and brain genes on the human and mouse X chromosomes. It is still unknown whether the X chromosome accrued these genes during its evolution or whether genes that find themselves on the X chromosome evolve such roles. ARX was originally autosomal in mammals and remains so in marsupials, whereas in eutherian mammals it translocated to the X chromosome. In this study, we examined autosomal ARX in tammars and compared it with the X-linked Arx in mice. We detected ARX mRNA in the neural cells of the forebrain, midbrain and hindbrain, and olfactory bulbs in developing tammars, consistent with the expression in mice. ARX was detected by RT-PCR and mRNA in situ hybridization in the developing tammar wallaby gonads of both sexes, suggestive of a role in sexual development as in mice. We also detected ARX/Arx mRNA in the adult testis in both tammars and mice, suggesting a potential novel role for ARX/Arx in spermiogenesis. ARX transcripts were predominantly observed in round spermatids. Arx mRNA localization distributions in the mouse adult testis suggest that it escaped meiotic sex chromosome inactivation during spermatogenesis. Our findings suggest that ARX in the therian mammal ancestor already played a role in male reproduction before it was recruited to the X chromosome in eutherians.

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Introduction


Arx is expressed at all stages of pancreatic development in the proliferating epithelium, differentiating pancreatic precursors, and islets of Langerhans in mice (Collombat et al. 2003). Deficiency of Arx results in a...
loss of mature endocrine α-cells with a concomitant increase in the numbers of β- and δ-cells (Collombat et al. 2003), suggesting that Arx is required for the specification and differentiation of α-cells (Collombat et al. 2003, 2005). Arx is strongly expressed in differentiating embryonic muscle and is progressively decreased during myogenesis, acting as a positive regulator of the differentiation and specification of skeletal muscle fibers (Biressi et al. 2008).

ARX is also important for mammalian gonadal development. In mice, Arx is strongly expressed in the fetal testis, principally in the interstitial cells, including peritubular myoid cells, tunica albuginea, endothelial cells, and interstitial fibroblast-like cells. However, Arx is barely detectable in developing Sertoli cells, Leydig cells, and germ cells (Kitamura et al. 2002). Arx-knockout neonatal male mice have smaller testes and seminiferous tubules of a larger diameter, but do not survive to adulthood (Kitamura et al. 2002). Arx functions through a paracrine signaling pathway to promote the differentiation of Leydig cells. Loss of Arx decreases the number of Leydig cells that produce testosterone to maintain testis development, resulting in X-linked lissencephaly with abnormal genitalia (XLAG) syndrome (Ogata et al. 2000, Kitamura et al. 2002). Surprisingly, there have been no detailed studies of ARX expression in the adult testes of any mammal. In addition to its role in testis development, weak expression of Arx is also observed in the developing mouse ovary at the boundary of the mesonephros at E14.5 (Kitamura et al. 2002), but a specific role for Arx in ovary development has not been investigated yet.

The X and Y chromosomes have evolved from autosomes in the common mammalian ancestor (Ohno 1967, Skalketsky et al. 2003, Ross et al. 2005, Graves 2006). The human X chromosome comprises an X conserved region (XCR) present in all therian mammals and recently added regions (XAR) present on the X chromosome in eutherians but located on an autosome in the common therian ancestor and in extant marsupials (Alsop et al. 2005). Comparative mapping in tammars, platypuses, chickens, and fish indicates that the XAR was translocated to the human X chromosome in a block of genes, after the divergence of marsupials from eutherian mammals (Graves 2008, Veyrunes et al. 2008, Delbridge et al. 2009). ARX maps to the XAR in tammars and is an X-linked in eutherian mammals while being autosomal (on chromosome 5p) in marsupials (Delbridge et al. 2008) and other non-mammalian vertebrates. Since the X chromosome is hemizygous in males, any genes that confer a reproductive advantage can be directly selected for. As a result, the eutherian X chromosome is thought to have become enriched with genes that have functions related to reproduction (Saifi & Chandra 1999, Wang et al. 2001, Vallender & Lahn 2004, Ropers & Hamel 2005, Delbridge & Graves 2007, Mueller et al. 2008).

As marsupials occupy an important position in X chromosome evolution in mammals, this study examined the expression of autosomal ARX in the tammar wallaby and compared this with the expression of its X-linked homologue Arx in the mouse. We demonstrate that autosomal ARX was already dynamically expressed during testis development and spermatogenesis before it was recruited to the X chromosome in eutherian mammals.

Materials and methods

Animals

Tammar wallabies (Macropus eugenii) from Kangaroo Island (South Australia) were maintained in open grassy yards supplemented with fresh vegetables and water ad libitum in our breeding colony in Melbourne, Australia. Pouch young of various ages were removed from their mother’s pouch for sampling. The age of pouch young was estimated using head length from published growth curves (Poole et al. 1991). Samples of mouse (Swiss white) tissue were collected opportunistically from other ethically approved experiments. Mice were bred in the Department of Zoology. All sampling techniques and tissue collection procedures conformed to the Australian National Health and Medical Research Council guidelines (2004) and were approved by the University of Melbourne Animal Experimentation and Ethics Committees.

Tissues

Adult tissues from tammars including brain, pituitary, hypothalamus, olfactory bulb, thyroid, muscle, spleen, mammary gland, heart, lung, liver, kidney, and adrenal were collected from three adult females (along with uterus and ovary) and two adult males (along with testes). Fetal gonads 1 day before birth (day 25 of gestation, n = 3), pouch young gonads after birth (dpp = days post partum), d2pp (n = 3), d7pp (n = 3), d10pp (n = 2), d15pp (n = 2), and d24pp (n = 3), and mature gonads (n = 2), as well as adult testes from mice (n = 4) for molecular analysis were snap-frozen in liquid nitrogen and stored at −80 °C until use. Olfactory bulbs were collected from both sexes of pouch young aged between d14 and d89pp. All tissues were collected under RNase-free conditions. Tissues for in situ hybridization were fixed overnight in 4% paraformaldehyde, washed several times with 1× PBS, and stored in 70% ethanol before paraaffin embedding and sectioning at 7 μm.

Cloning of tammar ARX gene and gene structure determination

Cross-species primers (ARXFw1 and ARXRv1) spanning the conserved regions of ARX were designed to partially clone ARX in tammars. Tammar ARX gene-specific primers (nested primers) were designed from the above PCR fragment for 3′ and 5′ RACE to isolate the full gene transcript (the sequences of all primers are listed in Table 1). cDNA template was derived from reverse-transcribed total RNA from the head of a fetal tammar, using the SMART cDNA Library Construction Kit
Table 1: Primers designed for the analysis of ARX expression by PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Application</th>
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<tbody>
<tr>
<td>ARXFW1</td>
<td>CAGTTTGAGTCTGTAATCCGAAGGAGCTTTTT RT-PCR</td>
<td>Cross-species cloning and RT-PCR</td>
</tr>
<tr>
<td>ARXRV1</td>
<td>TTTCCCAAGGAGGTGCAAGGGTATTGGAGGGCAAGTCT RT-PCR</td>
<td>Cross-species cloning and RT-PCR</td>
</tr>
<tr>
<td>ARXRV2</td>
<td>ACTCACCTCAGGGTGTTCGGGGTTTT TC</td>
<td>3’ RACE</td>
</tr>
<tr>
<td>SMART IV</td>
<td>AAGCAGTGGTATCAACGGGACTCAGGTATTACGTCGGCGGGTTTT GCAGGAGGGAAAGGACAGG RT-PCR</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>CDS III</td>
<td>ATTTAGAGGAGGGCCAGGGGCCAGCATGdT130N129T N</td>
<td>3’ RACE</td>
</tr>
<tr>
<td>5’ PCR primers</td>
<td>aagcagtggtatcaacgagaggactcaggtattacgaggccggctttgtgccaggtttttgttgc (n=A, C, or T; N=129=A, G, or C)</td>
<td>5’ RACE</td>
</tr>
<tr>
<td>ARXFW3</td>
<td>ACTACCTCAGGGTGTTCGGGGTTTT GCAGGAGGGAAAGGACAGG RT-PCR</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>ARXRV3</td>
<td>TGAGGAGGAAAGGACAGG RT-PCR</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>18S F</td>
<td>GATCCATTTGGGAAGGGCAAAGTCT</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>18S R</td>
<td>CCAAATCCACTACGGGACTTTCGGTTTT GCAGGAGGGAAAGGACAGG RT-PCR</td>
<td>RT-PCR</td>
</tr>
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</table>

*All the primers are listed in the 5’ to 3’ orientation; CDSIII, Smart IV, and 5’ PCR primers were synthesized by SIGMA (Genosys) according to the SMART cDNA Library Construction Kit (Clontech).* (Clontech). 5’ RACE was first carried out with primer 5’ PCR and ARXRV1 and then 5’ PCR and nested primer ARXRV2 were used to carry out 5’ RACE. 3’ RACE was carried out using the ARXFW1 and CDS III primers. Nested PCR was carried out using the ARXFW2 and CDS III primers. PCR cycling conditions were as follows: 35 cycles of 30 s, 95 °C; 60 s, 50 °C; and 90 s, 72 °C, in a 25 μl reaction mixture with GoTaq Green Master Mix (Promega) and 0.4 μM of each primer.

Comparative analysis was carried out to determine the gene structure of ARX in tammars using the gene structure of humans, mice, rats, South American grey short-tailed opossum (*Monodelphis domestica*), and the platypus (*Ornithorhynchus anatinus*) (http://www.ensembl.org/). Partial genomic sequence of *M. eugenii* was obtained from the trace archives at NCBI (http://www.ncbi.nlm.nih.gov/) to confirm the number of exons and their length and define the position of introns.

### Sequence alignment analysis

### Non-quantitative RT-PCR
Total RNA was isolated using RNAwiz (Ambion, Inc., Austin, TX, USA) according to the manufacturer’s instructions. The quality and quantity of total RNA were verified by gel electrophoresis and optical density reading with a Nanodrop (ND-1000 Spectrophotometer, Wilmington, DE, USA). Total RNA of 2 μg was treated with DNase1 (Ambion, Inc.) for 30 min at 37 °C. Total RNA of 1 μg was reverse-transcribed using the SuperScript III kit (Invitrogen).

To check the expression pattern of ARX in developing tissues and adult tissues, PCR was carried out in a 25 μl reaction mixture with GoTaq Green Master Mix and 0.4 μmol of each primer (ARXFW3 and ARXRv3). Amplification conditions were as follows: 95 °C, 30 s; 53.5 °C, 30 s; and 72 °C, 120 s for 35 cycles (ARX) or 25 cycles (18S). Samples were analyzed on a 1.2 and 2% agarose gel for ARX and 18S respectively.

### RNA isolation and northern blotting hybridization
Total RNA was isolated from snap-frozen adult tissues with RNAwiz (Ambion, Inc.). The concentration and quality of total RNA were examined as described above. Equivalent amounts of RNA (about 12 μg) were subjected to electrophoresis in a 1% agarose gel containing formaldehyde (Sambrook & Russell 2001). Northern blotting was carried out according to standard methods. Hybridization was carried out at 42 °C in ULTRAhyb solution (Ambion, Inc.) with [α-32P]dCTP-labeled cDNA probe from exon 2 to the end of 3’ UTR.

### ARX mRNA in situ hybridization
Antisense and sense RNA probes were prepared separately in the exon 2 region and were about 680 bp in length. Probes were synthesized and labeled with digoxigenin-UTP using SP6 or T7 RNA polymerase. Tissues were fixed in 4% paraformaldehyde overnight at 4 °C, rinsed several times with 1× PBS, embedded in paraffin, and sectioned onto polylysine slides (Menzel-Gläser, Braunschweig, Germany). After dewaxing, the sections were washed several times with 1× PBS, glycine, Triton X-100, and triethanolamine buffer and were then immediately hybridized with probes at 42 °C. Hybridization signals were detected using anti-Dig alkaline phosphatase-conjugated antibody and visualized with NBT/BCIP chromogen, according to the manufacturer’s instructions (Roche GmbH). The sections were counterstained with 0.1% Fast Red (Aldrich Chemical Corp., Milwaukee, WI, USA).
Results

Tammar ARX gene is highly conserved among mammals

The tammar ARX gene was cloned using degenerate primers and RACE PCR (GenBank accession number GU369938). ARX mRNA has 2747 bp, consisting of five exons (Fig. 1A). The sequence was confirmed from the trace archives of the tammar (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the human ARX gene structure, in which the conserved homeobox region spans exon 2 to exon 4.

The tammar ARX gene encodes a protein of 552 amino acids, highly conserved with eutherian ARX (Fig. 1B). We compared the ARX proteins of humans, mice, tammars, and Xenopus to determine which regions were under strongest selection (Fig. 1C). ARX is especially conserved within the functional domains including the N-terminal octapeptide, known to repress transcriptional activity and act as a nuclear localization signals recognized by importin, the homeodomain with its characteristic helix-turn-helix DNA-interacting motif and the C-terminal aristaless transcription activation domain.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Opossum</th>
<th>Human</th>
<th>Macaque</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Xenopus</th>
<th>Zebrafish</th>
<th>Fly</th>
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<td>Tammar</td>
<td>93</td>
<td>85.8</td>
<td>85.3</td>
<td>85.1</td>
<td>85.2</td>
<td>78.1</td>
<td>65.5</td>
<td>55.7</td>
<td>20.0</td>
<td>18.6</td>
</tr>
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</table>

Figure 1 Characterization of tammar ARX. (A) The tammar ARX gene has five exons (black/white boxes) with a total open reading frame length of 2719 bp. The white portion of boxes indicates the UTRs and black portions indicate protein-coding sequences (CDS). The length of each exon is labeled above the box. The broken line between exons represents introns of unknown size, while intron 4 (the solid line between exon 4 and exon 5) has a length of 2621 bp. (B) Cross-species ARX protein sequence comparison. The highest identity (93%) was observed between tammars and opossums and the lowest identity was observed between mammals and invertebrates as expected. (C) ARX protein sequence alignment between humans, mice, tammars, and Xenopus. The octapeptide (dashed box), nuclear localization signals (NLS, black underline), homeodomain (dashed box), fourth polyalanine (p(A)4 dashed box), and aristaless domain (dashed box) are highly conserved in all the species. The first and third p(A) are highly conserved in humans, mice, and tammars. The amino acids shaded black are identical across all the species.
ARX has a novel role in spermatogenesis

Tammar ARX revealed highly conserved and novel sites of expression

ARX plays critical roles in development of the forebrain, olfactory bulb, and gonads during embryogenesis in mice. To examine tammar ARX gene expression, we carried out RT-PCR using various tissues.

Tammar ARX was widely expressed during embryogenesis (data not shown) and was cloned from the developing brain. We detected ARX expression throughout gonadal development (Fig. 2). ARX was detected in the gonads before sexual differentiation (2 days before birth), during the time that testicular cords begin to form in males (around day 2 pp) and when the cortex and medulla form in the ovary in females (around day 7–8 pp), and later during germ cell proliferation in the ovary (on days 2–20 pp) and when the ovarian germ cells begin to enter meiosis (around day 25 pp) (Renfree et al. 1996). There was no obvious difference in ARX expression between male and female samples at any time point (Fig. 2), suggesting that it may be important in sexual differentiation of both sexes.

Using RT-PCR and northern blotting, we examined ARX expression in various adult tissues and confirmed the broad expression pattern in the brain observed in a previous report on the tammar (Delbridge et al. 2008). A northern blot confirmed that there was only one transcript of ~2.8 kb in adult testis (Fig. 3), in contrast to the three isoforms detected in human skeletal muscle (Stromme et al. 2002).

Interestingly, ARX was most strongly expressed in the adult testis, but, by comparison, it was only weakly expressed in the olfactory bulb, ovary, and skeletal muscle (Fig. 3). This finding suggests a previously unreported potential role for ARX in the adult testis. Therefore, we compared ARX mRNA localization and protein distribution in the adult testes of a marsupial and a eutherian mammal.

Tammar ARX maintains a conserved localization in the developing brain and gonads

ARX plays an important role in brain development in humans, mice, and zebrafish. To examine ARX expression and localization within the developing brain in marsupials, we examined the head on the day of birth, a stage that is equivalent to early intra-uterine development period of most eutherian mammals (Fig. 4A and B).

ARX mRNA was detected in the epidermis (Fig. 4C) surrounding the head, and strong expression was detected in the tongue and palate epithelium (Fig. 4D and E), as well as in the nasal epithelium. ARX mRNA staining was also detected in the olfactory bulb (Fig. 4F) and the vomeronasal organ (VNO) (Fig. 4G) and, using RT-PCR, strongly in the developing olfactory bulb (data not shown). Tammar ARX was expressed in the neuronal cells at the site of the trigeminal ganglion (Fig. 4H), telencephalon (Fig. 4I), mesencephalon, metencephalon (Fig. 4J), and myelencephalon (Fig. 4K) in a pattern similar to that observed in mice and zebrafish.

We examined ARX mRNA distribution in the developing gonads of tammar wallabies from before birth to 26 days after birth with mRNA in situ hybridization (Fig. 5). Germ cells in the tammar wallaby testis are still actively mitotically proliferating in the postnatal testis, peaking in number on day 25 pp. At this stage, the germ cells are developing gonocytes and are not yet spermatogonia (Tyndale-Biscoe & Renfree 1987). Before sex determination, tammar ARX was localized in the cytoplasm of germ cells (Fig. 5A and B). Two days after birth, during the formation of testicular cords, ARX was detected in the cytoplasm of somatic cells and germ cells.
(Fig. 5C and D). With testicular cord formation, ARX was expressed in the cytoplasm of some developing Sertoli cells and in germ cells (Fig. 5E). During ovarian differentiation (Fig. 5F), it was also cytoplasmic in the germ cells and some somatic cells. Around 25 days after birth, when male germ cells began to enter mitotic arrest, ARX was similarly detected in the cytoplasm of some Sertoli cells and germ cells (Fig. 5G). When female germ cells began to enter meiosis about day 25 after birth, ARX was observed predominantly in the cytoplasm of germ cells as well as somatic cells (Fig. 5H).

**Dynamic regulation of tammar ARX during spermatogenesis in the adult testis**

ARX mRNA was abundant in spermatogenic cells at specific stages and in some somatic cells (Fig. 6). Interestingly, unlike in the developing gonocytes, there was no expression in the mitotic spermatogonia and expression was only observed in these cells after the initiation of meiosis (Fig. 6A, B, C, D, and E). ARX mRNA was especially abundant in spermatocytes (Fig. 6A). As meiosis proceeded, it became restricted to the newly
formed round spermatids, but there was no staining in the spermatocytes (Fig. 6B). As round spermatids developed, ARX staining became weaker in the cytoplasm, but it was still strong in the round spermatids (Fig. 6C). No mRNA staining was detected in the elongated spermatids and spermatozoa (Fig. 6D and E), but there was still strong expression in the cytoplasm of round spermatids at these stages (Fig. 6D and E). ARX mRNA was also observed in some Leydig cells (Fig. 6A) and peritubular myoid cells at early stages of the spermatogenic cycle (Fig. 6A, B, C, D, and E) before round spermatids were transformed into elongated spermatids. No mRNA expression was detected in Sertoli cells at any stage (Fig. 6B).

**Dynamic regulation of Arx during spermatogenesis in the adult mouse testis**

This study, to our knowledge, is the first report of ARX expression in any adult mammalian testis. To determine whether our findings in the tammar represent a conserved role of ARX in the adult testis, we examined Arx expression at the mRNA level in the adult mouse testis.

The Arx mRNA expression pattern in the mouse testis was similar to that in the tammar testis, especially in spermatogenic cells. There was no detectable staining in the spermatagonia and somatic cells (Fig. 7A, B, C, and D), but strong expression was detected in the primary and secondary spermatocytes as well as round spermatids (Fig. 7A, B, C, and D). At later stages of the spermatogenic cycle, there was very weak staining in the cytoplasm of elongated spermatids (Fig. 7A), but no mRNA staining was detected in the mature spermatozoa (Fig. 7B).

**Discussion**

Comparative analysis of ARX/Arx in tammars and mice suggests that it has a highly conserved expression pattern during spermatogenesis, despite its different chromosomal location in these two species (X-linked in mice vs autosomal in marsupials). This study is the first to demonstrate that ARX may have a highly conserved role in spermatogenesis in addition to its established roles in neurogenesis.

**Evolution and conservation of ARX**

ARX has a conserved five-exon gene structure across eutherian and marsupial mammals and fruit flies. ARX is highly conserved at the protein level, with up to 93% identity between tammars and opossums, 85% with primates, and 56% with zebrafish (Fig. 1B). Conservation is even higher across the functional domains, such as the octapeptide domain, nuclear localization domain, homeodomain, and aristless domain from the amphibian *Xenopus* to eutherian mammals. As in the mouse, the tammar ARX has three nuclear localization signals (NLS) that are identical to those of other species, consistent with their conserved role in directing and importing ARX into the nucleus, possibly mediated by importin (Lin et al. 2009, Shoubridge et al. 2010). Thus, ARX has been highly conserved despite its physical relocation in the genome from an autosome to the XAR of the eutherian X chromosome (Delbridge et al. 2008).

ARX has conserved roles in early development. The strong mRNA staining of neural cells in the forebrain, midbrain, and hindbrain suggests that ARX plays a pivotal role in CNS development in tammars as it does in...
mice (Kitamura et al. 2002, Seufert et al. 2005). The similarity in tammar and murine ARX/Arx localization in the developing olfactory bulbs suggests that ARX is important for the normal development of this organ (Yoshihara et al. 2005). The mRNA distribution both in the olfactory epithelium and in the VNO suggests that ARX could be important for developing the sense of smell that is so essential for directing newborn wallabies into the pouch to locate the teats (Schneider et al. 2009).

Taken together, the conserved expression pattern in the developing brain of marsupials and mice suggests that ARX is a critical gene for brain development throughout therian mammalian evolution. Strong mRNA staining was also observed in the epithelium of the tongue and palate and in the underlying muscle. Early tongue development is especially important in the head of the newborn marsupial, which must be able to attach to the nipple and suck at the very early stages of development (Hughes & Hall 1988).

No obvious differences based on the RT-PCR were detected between the developing testis and ovary in tammar wallabies, similar to mice (Kitamura et al. 2002). The mRNA cellular location (Fig. 5) is similar to the pattern shown using whole-mount in situ hybridization of the mouse E11.5 testes (Colombo et al. 2004) and E14.5 gonads (Kitamura et al. 2002). Tammar ARX mRNA had a restricted pattern of expression in adult tissues with the highest expression in the adult testis and extremely weak expression in the olfactory bulb, adrenal, ovary, and muscle. By contrast, ARX is highly expressed in skeletal muscle in humans (Ohira et al. 2002, Stromme et al. 2002).

ARX escapes meiotic sex chromosome inactivation during spermatogenesis

This is the first report of the mRNA expression of ARX in the adult testis of any mammal during spermatogenesis. The spermatogenic cycle of the germ cells in the testis is coordinated and maintained by the somatic cells including the Sertoli cells, peritubular myoid cells, and Leydig cells that participate in spermatogenesis via their response to gonadotrophic signals (LH and FSH) from the hypothalamic–pituitary–testicular axis (Roser 2008, Yan et al. 2008, He et al. 2009, Li et al. 2009, Walker 2009, Cheng et al. 2010). However, most of the genes on the sex chromosomes are silenced during the meiotic phase of spermatogenesis by a process known as meiotic sex chromosome inactivation (MSCI), but are active in the mitotic stage and early meiosis (Hornecker et al. 2007, Horner et al. 2008).

mRNA in situ showing ARX localization in tammar adult testis. In situ hybridization of adult tammar testes with antisense ARX. mRNA distribution is indicated by dark blue staining and tissue is counterstained with 0.1% Nuclear Fast Red. Tammar ARX was strongly expressed in the round spermatids (RS) throughout all stages (A, B, C, D, E, F, and G) and there was no expression in the spermatogonia (SG), but there was stage-specific expression in other spermatogenic cells from the spermatocytes (SP) to the elongating spermatids (ES). At early stage I (A), ARX was expressed in the SP and some of the Leydig cells (LC) and peritubular myoid cells (PC) in the seminiferous cord. Slightly later in spermatogenesis at stage I (B), ARX was expressed in the RS and some of the LC, but in no other spermatogenic cells and Sertoli cells (SC). At stage IV (C), ARX was still strongly expressed in the RS, but weakly in the cytoplasm of ES. By stage V/VI (D), ARX was restricted to the RS. With the continuation of spermatogenesis (stage VIII, E), ARX was observed in the SP again besides the RS. At the early stage of spermatogenesis (F) and later stages (G), there was a dynamic expression of ARX in the LC but no expression in the SG in all the stages. The stages of spermatogenesis are described in Tyndale-Biscoe & Renfree (1987). SG, spermatogonia; SP, spermatocytes; RS, round spermatids; ES, elongating/elongated spermatids; SZ, spermatozoa; LC, Leydig cells; PC, peritubular myoid cells; SC, Sertoli cells. Scale bar = 100 μm.

Figure 6
ARX/Arx mRNA was mainly localized to the cytoplasm of SP and RS, but no expression was detected in SG and SC at stage II (A), Arx mRNA was also expressed in the cytoplasm of SP and RS, but no expression was detected in SG and SZ at stage X (C), Arx was stained in SP and weakly in ES, but no staining was found in SG and LC at stage XI (D), Arx was only detected in RS, but not in SG, ES, and SC. The stages of spermatogenesis are described in Hess & de Franca (2008). The abbreviations are the same as those in Fig. 6. Scale bar = 100 µm.

Our investigations also uncovered the previously unreported dynamic expression of ARX/Arx during spermatogenesis. In mice, mutations in Arx cause early postnatal death (Kitamura et al. 2002), making it impossible to examine the function of Arx in spermatogenesis in adults. A conditional knockout approach is needed to clarify this question. In non-traditional model species such as marsupials, functional analyses are extremely difficult and it is not possible at present to generate a transgenic or conditional transgenic model species such as marsupials, functional analyses are extremely difficult and it is not possible at present to generate a transgenic or conditional transgenic tammar. However, X-linked ARX/Arx mRNA is expressed in spermatocytes and round spermatids in both tammars and mice maintaining this profile of expression for over 160 million years of divergent evolution (Fig. 7). In addition, if mouse Arx is subject to MSCI, it would not be expressed at these stages in the germ cells of the adult testis. Our study provides strong evidence that Arx must escape MSCI. Thus, we postulate that the dynamic expression of Arx/ARX during spermatogenesis in both mice and tammars is highly suggestive of a potential role in spermatogenesis.

Conclusions

A similar but subtly different expression pattern during spermatogenesis between tammars and mice highlights the similarities in the basic processes of gametogenesis in marsupial and eutherian mammals. Although ARX is located on an autosome in tammars and not on the X chromosome as in eutherians, its expression does not appear to have been altered. This lends support to the theory that the mammalian X chromosome may selectively acquire SRR genes, rather than such genes evolving SRR functions after relocation to the X chromosome. Mouse Arx escapes MSCI during spermatogenesis, suggesting that it has a potentially important function in mammalian spermatogenesis that

Relationship of autosomal ARX in a marsupial with X-linked Arx in mouse

An important aim of this study was to understand the fundamental question of why a disproportionately high number of tests and brain genes have accumulated on the human and mouse X chromosomes. It is still unknown whether the X chromosome has accrued tests and brain genes during its evolution or whether genes that find themselves on the X chromosome evolve these roles. We examined how gene expression changed for the ARX gene when it became relocated from an autosome in the therian mammalian ancestor to the X chromosome in eutherians. ARX was originally autosomal in mammals and remains so in marsupials, making it an excellent comparison to the mouse X-linked Arx. Our findings suggest that ARX/Arx already played a role in male reproduction long before it was recruited to the X chromosome in eutherians, adding to the pool of data suggesting that the eutherian X chromosome actively recruits testis genes (Saifi & Chandra 1999, Wang et al. 2001, Vallender & Lahn 2004, Mueller et al. 2008).

Figure 7 Arx localization in the adult mouse testis. mRNA in situ hybridization of adult mouse testes with antisense mouse Arx. mRNA localization is indicated by dark blue staining and tissue is counterstained with 0.1% Nuclear Fast Red. At stage II (A), Arx mRNA was mainly localized to the cytoplasm of SP and RS and there was no expression in SG, PC, and LC; at stage VII (B), Arx was also expressed in the cytoplasm of SP and RS, but no expression was detected in SG and SZ; at stage X (C), Arx was stained in SP and weakly in ES, but no staining was found in SG and LC; at stage XI (D), Arx was only detected in RS, but not in SG, ES, and SC. The stages of spermatogenesis are described in Hess & de Franca (2008). The abbreviations are the same as those in Fig. 6. Scale bar = 100 µm.
has remained highly conserved for over 160 million years.

Declaration of interest

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

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