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—Original Article—

Localization of the Chromatin Remodelling Protein, ATRX in the Adult Testis

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Abstract. Mutations in ATRX (alpha-thalassaemia and mental retardation on the X-chromosome) can give rise to ambiguous or female genitalia in XY males, implying a role for ATRX in testicular development. Studies on ATRX have mainly focused on its crucial role in brain development and α-globin regulation; however, little is known about its function in sexual differentiation and its expression in the adult testis. Here we show that the ATRX protein is present in adult human and rat testis and is expressed in the somatic cells; Sertoli, Leydig, and peritubular myoid cells, and also in germ cells; spermatogonia and early meiotic spermatocytes. The granular pattern of ATRX staining is consistent with that observed in other cell-types and suggests a role in chromatin regulation. The findings suggest that ATRX in humans may play a role in adult spermatogenesis as well as in testicular development.
Key words: *ATR-X* syndrome, Germ cells, Spermatogenesis, Testis development

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The *ATR-X* (alpha thalassemia, mental retardation, X-linked) syndrome is a rare X-linked recessive developmental disorder affecting male children and young adults [1]. Affected individuals display a variable array of clinical features including severe mental retardation, a variety of facial and skeletal abnormalities, mild α-thalassemia, genital abnormalities, microcephaly and short stature [1].

The *ATRX* gene comprises 36 exons and encodes a modular protein of 280 kDa it contains two highly conserved domains, a PHD-like finger which interacts with chromatin, and a SWI//SNF-like ATPase domain [2]. A naturally occurring isoform that arises due to a failure to splice intron 11 from the primary transcript is also present, this isoform has been characterized as ATRXt and encodes a protein of roughly 150 kDa [3]. It shares a similar expression profile to ATRX, its function is unknown [3].

ATRX is a nuclear protein showing intense punctate staining associated with the DAPI-bright regions of the nucleus and interacts with the heterochromatin protein HP1 [4]. The protein is widely expressed throughout development is strongly associated with pericentromeric heterochromatin, ribosomal DNA repeats but also shows diffuse nuclear staining [4]. The ATRX protein is also found in promyelocytic leukemia nuclear bodies via an interaction with the Daxx protein and shows displays robust nucleosome remodeling activity, implying that ATRX functions as a chromatin remodeling protein [4,5]. The down regulation of alpha globin expression in *ATR-X* patients indicates that ATRX plays a role in gene expression [6]. Its specific cellular function is unknown.

In humans, 80% of mutations in *ATRX* cause genital abnormalities. These may be very mild (undescended testes or deficient prepuce), but the abnormalities extend through to hypospadia, micropenis and ambiguous or female genitalia (Table 1) [1, 7, 8]. The most severely affected children, who are clinically defined as male pseudohermaphrodites are most commonly associated with truncations of the C-terminus of the protein [1]. Histological analysis of the dysgenetic testis of an *ATR-X* patient revealed reduced numbers of seminiferous tubules bordered by Sertoli cells [7]. Leydig cells were present between the tubule structures but there was a complete absence of germ cells [7]. Histological examination of the testes of another patient revealed both to consist largely of loose interstitial stroma, with a few scattered spermatic tubules without lumina [9]. The tubules of the right testis contained only a few Sertoli cells, but a few spermatogonia remained in the left testis [9]. Both patients showed an absence of germ cells. The failure of the external genitalia of some *ATR-X* patients to fully masculinise and the severity of testicular dysgenesis suggests that affected testes fail very soon after testis determination. This would suggest that ATRX functions after sex determination and early testicular development and may be required for later testis deformation events [10]. To date, studies on ATRX have been focused primarily on its role in brain development and α-globin regulation. The function of ATRX in sexual differentiation is unknown. We describe the immunolocalisation of ATRX in wild-type human and rat testis in order to identify its potential roles and cellular sites of action in male gametogenesis.

Materials and Methods

Preparation of testis sections

ATRX protein expression was mapped by immunohistochemical evaluation of human and rat testis sections. Adult human testis biopsies fixed in Bouins and used in a previous study [11] were

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Table 1. Genital phenotype of patients with *ATR-X* syndrome [a]

Genital abnormality	Patients	Occurrence
Cryptorchidism	31	69%
Small penis	10	22%
Inguinal Hernia-hydrocoele	9	20%
Hypoplastic scrotum	9	20%
Hypospadias	8	18%
Small soft testes	8	18%
High lying/late testicular descent	6	13%
Deficient prepuce	6	13%
Female or ambiguous genitalia	5	11%
Shawl scrotum	2	4%

[a]adapted from [1].

obtained from Prof RI McLachlan. Samples of normal adult (70–90 day old) Bouin's fixed rat testis were provided by Dr L. O'Donnell. Animal experimentation was approved by the Monash Medical Centre animal ethics committee. Human testis tissue was embedded in paraffin wax and sections cut using standard histological procedures. Rat testis tissue was embedded into a low-melting temperature polyester wax and sections prepared as described previously [12].

Testis sections were de-waxed before re-hydrating through decreasing concentrations of ethanol (for polyester wax) or xylene/ethanol (for paraffin wax). For antigen retrieval, slides were microwaved in 1 × PBS (0.05M EDTA) for at 60 C for 5 min. Endogenous peroxidases were blocked by incubation with 1% hydrogen peroxide. Slides were then blocked in 1% normal goat serum in 1 × PBS (0.1% BSA) for 1 hr at RT and incubated overnight at 4 C with purified rabbit IgG or purified ATRX polyclonal antibody (H:300, Santa Cruz; 1:250) in 1 × PBS (0.1% BSA). Slides were incubated with Goat anti-Rabbit IgG antibody (1:500 abcam 64256) in 1 × PBS (0.1% BSA), for 1 hr at RT and then in ABC immunostain solution (DAKO) for 30 min at RT. AEC+ chromogen (Santa Cruz) was applied to the sections for colour development. The sections were counter-stained with haematoxylin for visualisation of nuclei, before mounting in Ultramount (DAKO).

Western blot analysis

Cell-lines NT2D1 and TM4 (corresponding to Sertoli-derived mouse cell-line) and total normal rat testis were homogenized in RIPA buffer corresponding to (150 mM NaCI; 50 mM Tris-HCI, pH 7.5; 0.25% sodium deoxycholate; 0.1% Nonidet P-40; protease inhibitor cocktail (Roche)) and approximately 5 μg of total protein was run in 3–8% TAE gradient gels (Invitrogen) and transferred to nylon membrane (Immobilon-P; Millipore Corporation). Membranes were incubated with a rabbit polyclonal anti-ATRX antibody, (H300) at 1:1000 dilution and subsequently with HRP-conjugated sheep anti-rabbit antibody (1:1000; Dako), the membrane was also incubated with a mouse monoclonal anti-ATRX antibody, (39f) at 1:10 dilution and subsequently with HRP-conjugated rabbit anti-mouse antibody (1:1,000; Dako). The 39f monoclonal antibody was produced by immunization of a 26-kDa fusion protein (residues 85–320) of the ATRX protein into BALB/c mice as previously described [4]. Signals were detected by using enhanced chemiluminescence (ECLplus; GE Healthsciences) and captured on X-ray film (Kodak).

Imaging and photocapture

Testis sections were examined under a contrast light microscope (Olympus Optical IX81 Fluoview Laser Scanning Confocal). Images were captured on computer with the associated camera (Olympus U-LH100HG), and using the IX2-BSW software.

Results

Immunolocalization of ATRX in normal adult human and rat testis

In normal adult human testis, strong ATRX staining was observed in the nuclei of Sertoli and peritubular myoid cells, and speckled, nuclear staining in the cells of the interstitium including Leydig cells (Fig. 1). In addition to the somatic cells, ATRX staining was also observed in developing germ cells in the early stages of spermatogenesis. In the monkey and human, there are two morphologically distinct type A spermatogonial sub-types; type Adark (Ad) and Apale (Ap) [11]. ATRX protein was observed in both Ad and Ap spermatogonia, leptotene/zygotene spermatocytes, and early meiotic pachytene spermatocytes (Fig. 1a–e). ATRX protein is also observed in the cytoplasm rather than nucleus in some type A pale spermatogonia and no expression in the vacuole that is characteristic of Ad spermatogonia (Fig. 1). Staining was no longer apparent in larger pachytene spermatocytes, or in post-meiotic cells, indicating that protein expression ceases during meiotic progression (Fig. 1a–e). ATRX was also observed in vascular endothelial cells (Fig 1b). To confirm that the ATRX antibody raised specifically in humans detects the rat orthologue, Western blot analysis was undertaken (Fig. 2). The H300 antibody (raised to the C-terminus of the protein) detected the rat orthologue of Atrx similar in size to both the human embryonal carcinoma cell line, NT2/D1 (human) and the Sertoli derived cell-line, TM4 (mouse) (Fig. 2a). In addition, the monoclonal antibody 39f raised against the N-terminal ADD domain detected both ATRX isoforms (Fig. 2b).

The staining pattern of Atrx in the rat testis was similar to that in human testis. Speckled nuclear staining was observed in Sertoli, Leydig and peritubular myoid cells, and Type A spermatogonia and early spermatocytes (preleptotene, leptotene, zygotene and early pachytene) (Fig. 3a–d). Staining disappeared during the pachytene spermatocyte phase, when homologous chromosome recombination occurs, and no Atrx staining was observed in late pachytene spermatocytes (Fig. 3c) and post-meiotic spermatids (Fig. 3a–c). As in humans, ATRX was also observed in the vascular endothelial cells (Fig 3b and d). Rat testis sections incubated with rabbit IgG showed non-specific and generalized cytoplasmic staining; highlighting the specificity of the observed nuclear ATRX staining (Fig. 3e–f). The granular staining pattern of ATRX, together with the fact that ATRX is a chromatin remodeller is consistent with the proposition that ATRX colocalizes with chromatin [2, 3].

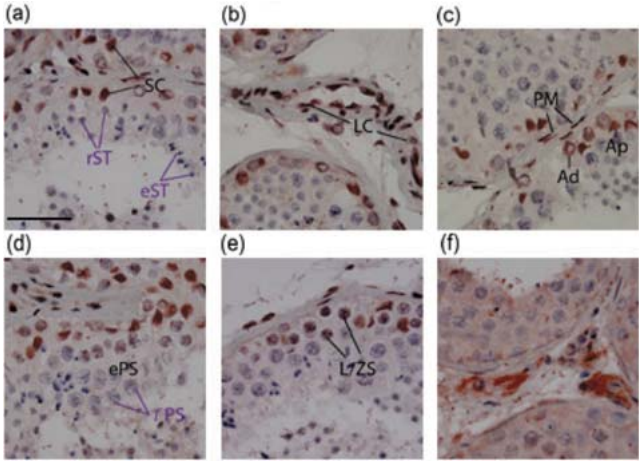

Fig. 1. Immunohistochemical staining of ATRX in normal adult human testis (panels a–e). Using a polyclonal (rabbit) anti-ATRX antibody (H300, Santa Cruz), strong staining (indicated in brown) is observed in the nuclei of Sertoli cells (SC), peritubular myoid cells (PM) and interstitial cells including Leydig cells (LC). ATRX is also present as nuclear speckles in early germ cells including; Type A dark (Ad) and pale (Ap) spermatogonia leptotene (LS) and zygotene (ZS) spermatocytes and early pachytene spermatocytes (ePS). Later-staged germ cells such as late pachytene spermatocytes (lPS) and round (rST) and elongated (eST) spermatids were negative for ATRX staining (indicated in purple). The IgG control (panel f) shows non-specific, cytoplasmic staining. All images are representative of 3 independent experiments using tissue from 3 different individuals (Scale bar=200 μm).

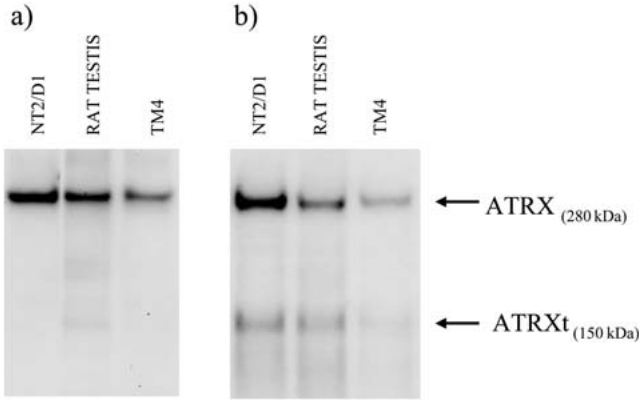

Fig. 2. Detection of full-length and truncated isoforms of the ATRX protein. Detection of full-length and truncated isoforms of ATRX using human ATRX antibodies as determined by Western blot. 5 μg of rat testis, NT2D1 and TM4 cell extract was resolved on a 3–8% Tris-Acetate gel and probed with a) the polyclonal anti- human ATRX antibody (H: 300, Santa Cruz) and b) monoclonal antibody 39f. Arrows indicate full-length protein and truncated isoform.

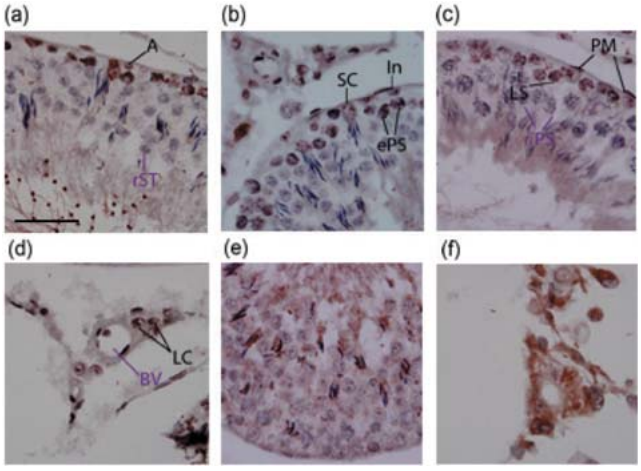

Fig. 3. Immunohistochemical staining of Atrx in normal adult rat testis (panels a–d). Granular ATRX staining (indicated in brown) was observed in the nuclei of Sertoli cells (SC), peritubular myoid cells (PM) and interstitial cells including Leydig cells (LC). Atrx is also expressed in the nucleus of early germ cells including; Type A spermatogonia (A), intermediate spermatogonia (In), leptotene spermatocytes (LS) and early pachytene spermatocytes (ePS). Later-staged germ cells such as late pachytene spermatocytes (lPS) and round (rST) and elongated (eST) spermatids were negative for ATRX staining (indicated in purple). The negative (IgG) control is shown in panels e and f; a diffuse and generalized cytoplasmic staining was observed in all stages. All images are representative of 3 independent experiments using tissue from 3 or more different animals. (BV= blood vessel) (Scale bar=200 μm).

Discussion

This study shows that ATRX protein is present in the adult testis of rats and humans. Speckled, nuclear staining of ATRX was observed in the somatic cells of the adult testis, in Sertoli and peritubular myoid cells, as well as in the cells of the interstitium including Leydig cells. Similar staining has been found in the developing mouse and wallaby testis (A.Pask, pers. comm). The study also indicates that ATRX protein is expressed in germ cells during the early phases of spermatogenesis. Expression was observed in spermatogonia (both Ap and Ad in humans), leptotene, zygotene and early pachytene spermatocytes. ATRX protein disappeared during the pachytene phase of spermatocyte development and was not observed in spermatocytes during the final meiotic divisions, or in post-meiotic haploid spermatids. The granular staining pattern observed in ATRX-positive cells suggests that ATRX colocalizes with heterochromatin in the adult human and rat testis, and is concordant with various studies reporting ATRX colocalisation with heterochromatin in transcriptionally quiescent mouse oocytes, and during interphase and mitosis in HeLa cells [4, 14].

The wide, nuclear distribution of ATRX in both somatic and germ cell types of the testis indicates that ATRX plays a role in the adult testis, in both somatic and germ cells. The strong staining of ATRX in Leydig cells, and the observation that ATRX patients with male pseudohermaphroditism fail to produce normal amounts

of testosterone suggests that ATRX may be involved in Leydig cell steroidogenesis [7, 15]. Our observations in the rat and human testis are largely concordant with the study in mouse spermatogenesis; however, while they report no ATRX expression in pachytene spermatocytes [16], we observe distinct nuclear staining of ATRX in early pachytene spermatocytes but not in late pachytene spermatocytes.

The expression pattern of ATRX in the vascular endothelial cells is not surprising since ATRX is expressed in many cell types during development, in addition one of the major phenotypes in *ATR-X* syndrome is the blood disorder alpha-thalassemia [1, 17, 18].

Studies have shown an association of ATRX with pericentromeric heterochromatin during mitosis and previous studies have shown that ATRX, as part of a multi-protein complex, contains diverse chromatin remodelling activities [4, 5, 19]. While these studies suggest ATRX to be a general component of chromatin remodelling complexes, the phenotypes of *ATR-X* syndrome patients emphasizes additional and specific roles for the ATRX protein in certain tissues. This is observed in several mouse model studies that show an essential role in brain development [17, 20]. We propose that, within the testis, the specificity of gene regulation by ATRX could be conferred by interaction with testis-specific transcription factors—possibly through the C-terminus of the protein, especially since truncations in this region result in pseudohermaohrodism [9]. Alternatively, ATRX may have a general house-keeping function in the maintenance of the adult testis and spermatogenesis, but a different and more specific role during the critical stages of sex differentiation during embryogenesis. This is not an unusual feature of a gonad. Many genes such as WT1 and SF1 have specific roles in early gonad differentiation and different critical roles in the mature gonad (reviewed by [21]).

During gametogenesis in the female, ATRX is required for correct chromosome alignment during metaphase II of meiosis in mouse oocytes [14]. The nuclear localization of ATRX during mouse meiosis appears to be sexually dimorphic, and implies different roles for ATRX during male and female gametogenesis. In mice, ATRX expression appears to arrest before the pachytene phase of prophase I in male germ cells while remaining visibly associated with pericentromeric chromatin for the duration of meiosis I and II in oogenesis [16]. These results suggest that while being crucial to chromosome segregation in oogenesis, ATRX is unlikely to play a direct role in metaphase II of male meiosis.

Interestingly, a separate study in cultured human cells and embryonic mouse brain also found ATRX to be important for proper chromosome segregation during mitosis [22]. Thus a role is emerging for ATRX in maintaining meiotic/mitotic integrity during oogenesis and in somatic cells. The results provided in this study are consistent with a role for ATRX in male germ cell mitosis and meiosis. A high level of expression was detected in Sertoli cells which do not undergo mitosis, and are terminally differentiated. This also occurs in other cell types most notably in erythopoeitic cell-lineage [18]. It is likely therefore that ATRX has quite diverse roles in the testis including a potential role in steroidogenesis and Sertoli cells function as well as a role in mitosis/meiosis.

The precise role of ATRX during gonadal differentiation is still unknown. Complete *Atrx* knockout mouse embryos die of extraembryonic defects around embryonic day 8.5, before the onset of testicular morphogenesis [23]. Hence a conditional, Atrx-gonad-specific knock-out mouse model would allow for a better understanding of the role of ATRX in sex differentiation. These findings suggest that ATRX acts within multiple cell lineages of the testis. Similar to its role in ovarian function, ATRX may regulate at least the early stages of gametogenesis in human and rat testes; however, ATRX may also participate in somatic cell function, such as the regulation of adult Sertoli and Leydig cell function. The granular pattern of ATRX staining is consistent with colocalisation with pericentromeric chromatin, suggesting that it may act as a regulator or coordinator of gene expression within several cell lineages of the testis.

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