

Stage-dependent expression of extra-embryonic tissue-spermatogenesis-homeobox gene 1 (ESX1) protein, a candidate marker for X chromosome-bearing sperm

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Abstract. Extra-embryonic tissue-spermatogenesis-homeobox gene 1 (*Esx1*) encodes an X-linked homeobox protein. Despite the fact that the temporal and spatial mRNA expression pattern of the protein has been studied extensively in the testis, specific localisation of ESX1 in the testis remains to be determined. In the present study, we generated ESX1 antiserum to investigate the stage- and tissue-specific expression of ESX1 in the mouse. Western blotting and immunofluorescent analyses revealed that general localisations of ESX1 were consistent with its RNA expression patterns; that is, it was restricted mainly to the placenta and testis. Immunofluorescent studies demonstrated that ESX1 existed in the testes after 3 weeks of age, coincident with the appearance of round spermatids in the seminiferous tubules. Moreover, ESX1 expression became more abundant in the luminal regions of the seminiferous tubules as the development of round spermatids progressed into spermatozoa. In contrast, reduced expression of ESX1 was observed in experimentally induced cryptorchid testes. The later expression of ESX1 suggests a role in post-meiotic germ cell development. To further understand ESX1 expression in sperm with respect to X chromosome-bearing sperm, we used ESX1 antiserum to immunostain sperm by confocal laser microscopy. Approximately half the sperm population was recognised by the ESX1 antiserum. On the basis of results of the present study, we suggest that ESX1 could be used as a protein marker for X chromosome-bearing sperm.

Extra keywords: protein marker, spermatogenesis, testis.

Introduction

Spermatogenesis is a highly specialised process of male germ cell differentiation, starting with spermatogonia and eventually resulting in two opposite-sexed populations of spermatids (Bellvé *et al.* 1977; Russel *et al.* 1990; Dym 1997; Eddy 2002). The X and Y chromosomes are separated from autosomes and form heterochromatic sex bodies during the meiotic prophase of spermatogenesis (Monesi 1965; Solari 1974; Hendriksen *et al.* 1995). After completion of the meiotic divisions, the sex chromosomes have a condensed pattern that is different from that of the autosomes and are assumed to be transcriptionally inactive (Sachs 1954; Monesi 1965; Sassone-Corsi 2002). However, contradictory observations indicate that many genes are still transcribed in post-meiotic spermatogenic cells (Hendriksen *et al.* 1995) and that their gene products constitute many cytoplasmic components within intercellular bridges connecting the differentiated male germ cells (Dym and Fawcett 1971; Li *et al.* 1997). The post-meiotic transcription of genes raises the possibility of the specific expression of X or Y chromosomal genes localised in round spermatids and cytoplasmic

fragments (Hendrikson *et al.* 1995). Among the candidate genes that have been implicated in testicular post-meiotic germ cell development are *Xist*, *CREMtau*, *p63*, *SREBP2 gc* and extra-embryonic tissue-spermatogenesis-homeobox gene 1 (*Esx1*, also known as *Spx1*; Dolci *et al.* 1994; Poulat *et al.* 1995; Li *et al.* 1997; Li and Behringer 1998; Nakamuta and Kobayashi 2004; Wang *et al.* 2004). To examine the possibility of these gene products being used as markers for X chromosome-bearing sperm, we investigated *Esx1*. Previous results indicate that *Esx1* mRNA expression is restricted to a subset of extra-embryonic tissues and to pre- and post-meiotic germ cells in the adult testis (Branford *et al.* 1997; Li *et al.* 1997; Li and Behringer 1998). The *Esx1* gene, a member of the homeobox gene family, functions as a regulator of placental development and fetal growth (Li and Behringer 1998; Yan *et al.* 2000; Fohn and Behringer 2001; Singh *et al.* 2004). Targeted null mutation of *Esx1* in mouse embryonic stem cells generates a lethal homozygote (Li and Behringer 1998). Further studies have revealed that the fetal growth retardation in X^{*Esx1*}Y male and X^{*Esx1*}X female mutants was caused by the overgrowth and defective morphogenesis of the

labyrinth layer of the placenta. However, *Esx1* hemizygous mutant males were fertile, demonstrating that *Esx1* was not essential for spermatogenesis.

Based on differences in the physicochemical characteristics of X and Y chromosome-bearing sperm, physiologists have explored many candidate markers to identify opposite-sexed sperm (Koopman *et al.* 1990; Han *et al.* 1993). Current strategies include detecting the H-Y antigen cytogenetically, measuring X-linked enzymes and localising Y chromosome-specific DNA sequences (Krc0 and Goldberg 1976; Jafar and Flint 1996; Reubinoff and Schenker 1996; Howes *et al.* 1997; Blecher *et al.* 1999; Hendriksen 1999; Seidel and Johnson 1999; Johnson 2000; Gardon *et al.* 2004). Unfortunately, these technologies have not been applied consistently to identify sexed sperm in laboratory animals, other large domestic animal species or humans (Grabske *et al.* 1975; Vidal *et al.* 1993; Risopatron *et al.* 1996; Johnson and Welch 1999a; Kim *et al.* 1999).

In the present study, we suggest the ESX1 protein as a candidate marker to differentiate X and Y chromosome-bearing sperm. We performed a series of experiments to localise ESX1 expression in the testis and sperm. Meanwhile, other Y chromosome-specific antiserum was used to immunostain the sperm as a counter reference. The sex-determining region Y chromosome (*SRY*) gene, which contains a high mobility group box DNA-binding domain, is a transcriptional modulator in the sex-determining pathway (Capel *et al.* 1993; Rossi *et al.* 1993; Hacker *et al.* 1995; Poulat *et al.* 1995). Our analysis demonstrated that ESX1 antiserum could recognise half the mouse sperm population, whereas *SRY* antiserum stained positively with the other half of the population. These results suggest that ESX1 antiserum works as a useful agent in distinguishing X and Y chromosome-bearing sperm.

Materials and methods

Animals

Approximately 50 ICR inbred strain mice, aged from 1 to 18 weeks, were purchased from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan, ROC).

Induction of cryptorchidism

Surgery was performed according to previous reports (Sun *et al.* 1997; Kim *et al.* 1999; Danno *et al.* 2000; Shinohara *et al.* 2000; Rockett *et al.* 2001; Senoo *et al.* 2002; Barqawi *et al.* 2004). The mice, specifically at 4 weeks of age, were operated on for the induction of cryptorchidism. Briefly, at 4 weeks of age, mice were anaesthetised and a small incision was made in the midline abdomen to induce the cryptorchidism. Testes were anchored to the inner lateral abdominal wall by a suture passing through the connective tissue of the cauda epididymis. Animals were housed individually for 12 weeks after the operation and were killed by cervical dislocation.

Separation of the testicular germ cell populations and sperm preparation

Separation of spermatogenic cells was based on the centrifugal elutriation system, as described previously (Grabske *et al.* 1975; Bellvé *et al.*

1977; Meistrich *et al.* 1981; Mays-Hoopes *et al.* 1995; Odorisio *et al.* 1996; Marret and Durand 2000; Izadyar *et al.* 2002). Briefly, testes were excised, decapsulated and cut into small pieces. Testes were incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 1 mg mL⁻¹ trypsin, 0.75 mg mL⁻¹ collagenase, 1 µg mL⁻¹ proteinase inhibitor, 5 µg mL⁻¹ DNase I and 100 IU mL⁻¹ penicillium-streptomycin for 1 h at 37°C in a shaking water bath operated at 140 cycles min⁻¹. After washing with DMEM/F12 medium and filtering through 80- and 40-µm nylon filters, cell suspensions were subjected to centrifugal elutriation using an elutriator rotor (JE 5.0; Beckman Instruments, Palo Alto, CA, USA). Elutriation conditions were set according to the cell size and mass. The separating fractions of spermatogonia, round spermatids, secondary spermatocytes and primary spermatocytes were collected sequentially at 750g (60 mL min⁻¹), 420g (60 mL min⁻¹), 190g (40 mL min⁻¹) and 50g (40 mL min⁻¹) respectively. The enriched cell pellets were collected by centrifugation at 3000g for 10 min. The purity of these specific cell types was examined under a light microscope and were as follows: spermatogonia 85%, round spermatids 80%, secondary spermatocytes 76% and primary spermatocytes 89%.

Spermatozoa were collected from the cauda epididymis of mature male mice (Chomczynski 1992; Munne 1994; Howes *et al.* 1997; Kawarasaki *et al.* 1998; Welch and Johnson 1999). Briefly, the epididymal cauda was cut into small pieces in a 35-mm Petri dish to isolate epididymal spermatozoa. Spermatozoa were allowed to swim up for 30 min at 37°C, then the untreated semen and supernatant of the solution were centrifuged for 10 min at 300g to pellet the spermatozoa. Resuspended spermatozoa were air-dried on slides for further immunofluorescent analysis.

Generation of ESX1 antiserum

Generation of polyclonal ESX1 antiserum against the recombinant ESX1 homeodomain region was performed according to the methods of Yan *et al.* (2000). In the present study, polymerase chain reaction (PCR) was used to amplify the recombinant ESX1 homeodomain region (amino acids 183–248) flanked with the *Bam*HI and *Hind*III restriction endonuclease cut sites. After digestion with *Bam*HI and *Hind*III restriction endonuclease, the resulting fragment was subcloned into a plasmid pQE-9 vector (Qiagen, NY, USA). The recombinant ESX1 protein was expressed in *Escherichia coli* and used as an antigen to generate polyclonal ESX1 antiserum in New Zealand white rabbits. The specificity of the antiserum was checked by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 ng recombinant ESX1 was reacted with diluted ESX1 antiserum ranging from 1 : 100 to 1 : 100 000 000 for 1 h. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted 1 : 1000; Zymed Laboratories, San Francisco, CA, USA) was added to each reaction and incubated for 1 h. The chromogen ortho-phenylenediamine (OPD; Sigma, St Louis, MO, USA) was added to visualise activity. Plates were read with an ELISA reader (MrX; Dynatech Laboratory, Haverhill, MA, USA) at a wavelength of 495 nm.

Western blot analysis

Total protein (50 µg) from placenta, ovary, testis and testicular germ cell extracts was denatured and separated onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to a polyvinylidene fluoride membrane, blots were blocked with 5% non-fat skim milk in phosphate-buffered saline (PBS), subsequently incubated in ESX1 antiserum (diluted 1 : 5000) or *SRY* antiserum (diluted 1 : 5000; sc-8233; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then treated with HRP-conjugated goat anti-rabbit IgG (diluted 1 : 5000; Zymed Laboratories) or HRP-conjugated rabbit anti-goat IgG (diluted 1 : 5000; Zymed Laboratories). After washing in PBS containing 0.5% Tween-20 (PBS-T), peroxidase activity was visualised using the ECL Plus immunodetection system according to the

manufacturer's instructions (NEN™ Life Science, Boston, MA, USA). The same membrane was reprobbed with anti-actin antibody (diluted 1 : 5000; A-5316; Sigma) and HRP-conjugated goat anti-mouse IgG (diluted 1 : 5000; Zymed Laboratories) as a control for equal protein loading.

Immunofluorescence microscopy

Suspended spermatozoa were air-dried on slides and fixed in 4% paraformaldehyde/PBS for 1 h. Freshly dissected testis and placental tissues were rinsed in PBS and fixed in 4% paraformaldehyde/PBS for 3–12 h. After fixation, tissues were embedded and cryosectioned at 20 μ m for immunodetection analysis. Fixed tissue sections and spermatozoa were washed three times in PBS and then incubated with 5% bovine serum albumin in PBS for 30 min, followed by at least 12 h incubation with ESX1 antiserum (diluted 1 : 100) in tissue sections or ESX1 and SRY antiserum (diluted 1 : 100; sc-8233; Santa Cruz Biotechnology) in sperm in a moist chamber at 4°C. Slides were washed three times in PBS-T, followed by 1 h incubation in fluorescein-conjugated goat anti-rabbit IgG diluted 1 : 200 in tissue sections or Cy5-conjugated goat anti-rabbit and fluorescein-conjugated rabbit anti-goat IgG (diluted 1 : 200) in sperm at room temperature. Coverslips were washed three times in PBS-T and incubated with propidium iodide/PBS as a counterstain (diluted 1 : 500; Molecular Probes, Eugene, OR, USA), mounted on slides and photographed with an LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany). A three-dimensional view was composed from Z-section scanning images and analysed using the LSM 510 image system (Zeiss).

Statistical analysis

Data are presented as the mean \pm s.e.m. Statistical analysis was performed using Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

Spatial expression of ESX1 protein in mouse placenta and testis

To confirm the specificity of ESX1 antiserum, we checked the specificity of the antiserum using ELISA. The results showed that the rabbit anti-mouse ESX1 antiserum reacted with recombinant ESX1 protein specifically. Consequently, Western blotting and immunofluorescent analyses were performed to determine the expression of ESX1 in the placenta, ovary and testis. The size of the band shown in Western blot analysis was consistent with the estimated molecular weight of ESX1. In a preliminary experiment, the ESX1 antiserum was pre-incubated with recombinant ESX1 and assayed under immunofluorescent microscopy. No stained signals were found. Western blotting results indicated that ESX1 expression, consistent with its mRNA expression (Branford *et al.* 1997; Li *et al.* 1997; Li and Behringer 1998), was high in the placenta and testis (Fig. 1A). To determine the localisation of ESX1 in the placenta, immunofluorescent analysis was performed from Day 12 of mouse gestation. Consistent with the findings of Li *et al.* (1997) and Yan *et al.* (2000), the present data revealed that positively stained ESX1 signals appeared to be in the labyrinth layer of the placenta (Fig. 1B). We then performed Western blotting and

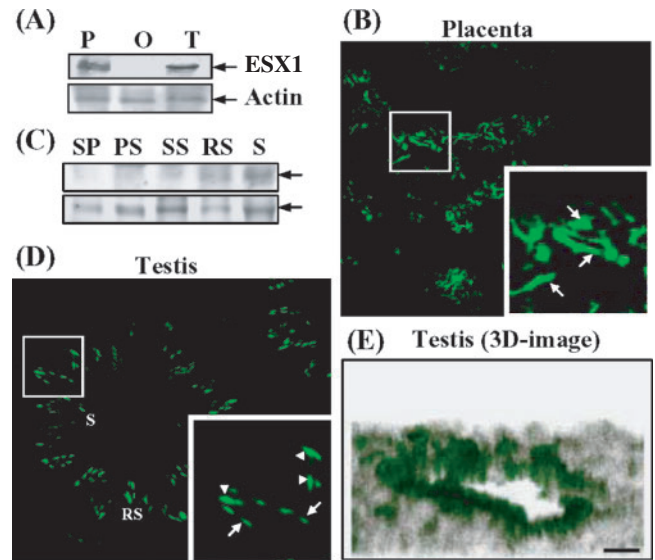


Fig. 1. The extra-embryonic tissue-spermatogenesis-homeobox gene 1 (ESX1) protein was localised predominantly in the placenta and testis. Western blot analysis shows that mouse ESX1 has a molecular mass of approximately 41 kDa, indicated by arrows (A,C; upper panels). The actin is used as a control of equal protein loading (lower panels). P, placenta; O, ovary; T, testis; SP, spermatogonia; PS, primary spermatocytes; SS, secondary spermatocytes; RS, round spermatids; S, sperm of testicular germ cells. The confocal micrographs show that ESX1 protein is expressed in placenta from Day 12 of gestation and in adult testis. (B) Low-power view of ESX1 protein expression in the subpopulation of the labyrinth layer of the placenta. The inset (lower right) is a twofold magnification of the boxed area. The intense ESX1 signals (green) were localised predominantly to the cytoplasm (arrows). (D) In the testis, a higher magnification of the boxed area (lower right) shows that round spermatids (arrows) and spermatozoa (arrowheads) have high levels of green fluorescence on the nuclei. (E) A top view of the three-dimensional confocal images shows that ESX1 protein (green fluorescence) was localised predominantly in the luminal region of the seminiferous tubule. Scale bar = 25 μ m.

immunofluorescent analyses to determine the localisation of ESX1 in mouse testicular germ cells. As shown in Fig. 1C,D, ESX1 was detectable in a subpopulation of round spermatids and was expressed intensively in spermatozoa. However, low levels of ESX1 were also detected in secondary spermatocytes. We suspect that the low signals were due to the presence of contaminating round spermatids in the elutriation fraction of secondary spermatocytes. Interestingly, the three-dimensional view composed from Z-section scanning images showed a similar expression pattern of ESX1 localising near the lumen of the seminiferous tubule (Fig. 1E).

Stage-specific locations of ESX1 protein during testicular development

During spermatogenesis, round spermatids first appeared at postnatal Week 3 and condensing spermatids were present at

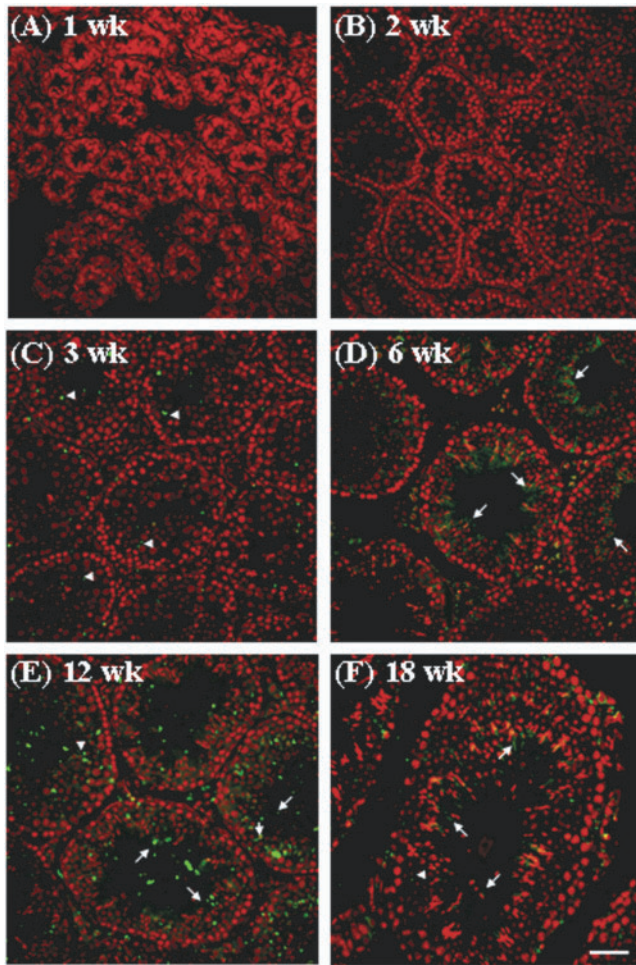


Fig. 2. Spatial and temporal localisation of extra-embryonic tissue-spermatogenesis-homeobox gene 1 (ESX1) protein during testicular development by immunofluorescent microscopy. Mouse testes are isolated from 1 (A), 2 (B), 3 (C), 6 (D), 12 (E) and 18 (F) weeks of age. The green fluorescence indicates the locations of ESX1 and the red colour represents sections counterstained with propidium iodine. The intense ESX1 expression in testes was localised predominantly to the round spermatids (arrowheads) and spermatozoa (arrows). Scale bar = 25 μ m.

Week 4 in the seminiferous tubules. Therefore, testes at several developmental stages were examined for ESX1 expression. No significant signals of ESX1 above background levels were detected in sections of testes isolated from mice before 3 weeks of age (Fig. 2A,B). Positive signals for ESX1 in round spermatids were first observed at 3 weeks of age (Fig. 2C). Large amounts of ESX1 signals were detected predominantly in a subpopulation of spermatids near the lumen of the seminiferous tubules (Fig. 2D,E). The expression of ESX1 increased in round spermatids and spermatozoa over 6 weeks of age and decreased at 18 weeks of age (Fig. 2D–F). We found that the high distribution of ESX1 in seminiferous tubules at stages IV–VII mirrored that of its corresponding

mRNA, as shown previously in the studies of Branford *et al.* (1997) and Li *et al.* (1997).

Furthermore, experimentally induced cryptorchidism, one of the well-characterised testicular injury systems, was performed in the present study to determine the specific localisation of ESX1 in the testis. Cryptorchidism has been shown to cause an increase in the apoptotic rate of germ cells and to reduce the number of mature germ cells in adult testis (Shinohara *et al.* 2000; Sassone-Corsi 2002; Rasoulopour *et al.* 2003; Barqawi *et al.* 2004). Our morphological observation of cryptorchid testis tissue supports the notion that germ cells are progressively diminished; only a few mature sperm remain in the defective testis over time (Fig. 3D–F). Decreased expression of ESX1 compared with control testicular sections (Fig. 3A–C) was seen in round spermatids and spermatozoa.

Specific localisations of SRY protein in the testis

Expression of the SRY protein was determined in the testis by Western blotting and immunofluorescent analyses. Consistent with the expression of SRY in adult testis (Rossi *et al.* 1993; Salas-Cortes *et al.* 1999; Salas-Cortes *et al.* 2001), Western blotting results showed that SRY was, indeed, present in the mouse testis (data not shown). In addition, higher-magnification observation of testicular sections using confocal laser microscopy indicated that intense SRY signals were localised in spermatids and Sertoli cells (Fig. 4). Our results demonstrate that SRY is specifically localised in the spermatids and Sertoli cells of the adult testis.

Distinct localisations of ESX1 and SRY in X and Y chromosome-bearing sperm

To investigate the distributions of ESX1 and SRY in mouse sperm, immunofluorescence confocal microscopy was performed using both ESX1 and SRY antisera. Interestingly, the results showed that almost half the sperm nuclei were detected using ESX1 antiserum (Fig. 5A), whereas the other half of the population was stained with SRY antiserum (Fig. 5B). As shown in Fig. 5C, all sperm were counter-stained with propidium iodine. A merged image shows the distinct locations of ESX1 and SRY in the two opposite-sexed sperm populations (Fig. 5D).

Use of both ESX1 and SRY antisera to recognise X and Y chromosome-bearing sperm separated by the swim-up technique

Our immunofluorescent analyses suggested that ESX1 and SRY antisera could recognise opposite-sexed sperm. To further investigate the efficacy of our antiserum in differentiating pre-selected sperm, the swim-up protocol, so far the most common procedure used in clinics, despite the controversy surrounding this technique, was performed to separate the

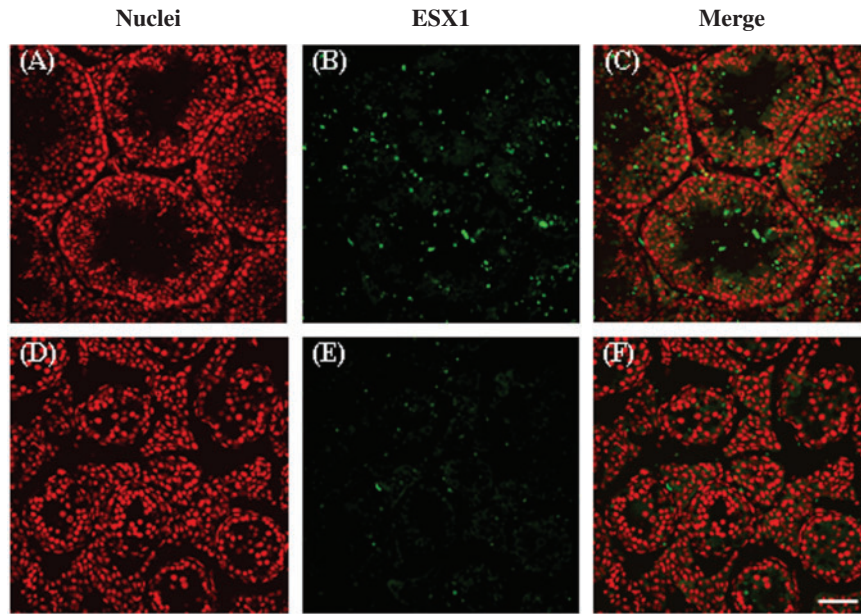


Fig. 3. Confocal micrographs show that extra-embryonic tissue-spermatogenesis-homeobox gene 1 (ESX1) protein expression is reduced in mouse testis with experimentally induced cryptorchidism. Green fluorescence indicates the location of ESX1 (B,E), whereas the red colour represents testicular sections counterstained with propidium iodine (A,D). (C,F) Merged images of (A) plus (B) and (D) plus (E) respectively. Scale bar = 25 μ m.

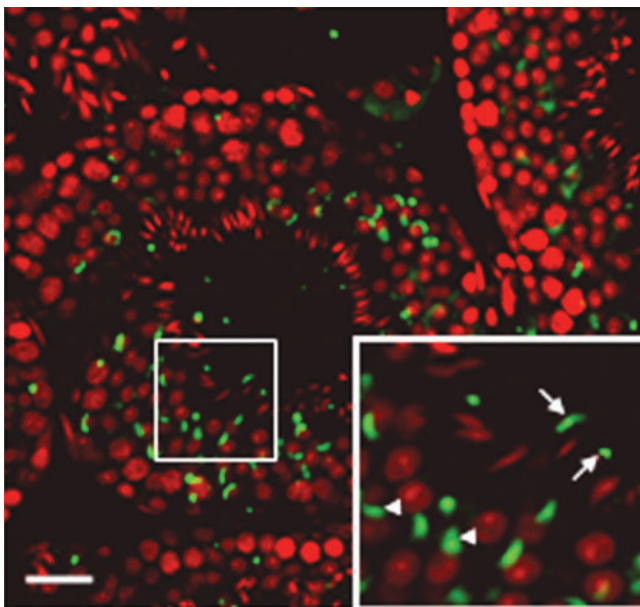


Fig. 4. The sex-determining region Y chromosome (SRY) protein was localised specifically in the testis. Confocal micrographs show SRY expression in the testicular section. Low-power view of SRY expression in the subpopulation of spermatids and Sertoli cells. The inset (lower right) is a twofold magnification of the boxed area. The intense SRY signals (green) were localised predominantly to the Sertoli cells (arrowheads) and spermatozoa (arrows). Scale bar = 25 μ m.

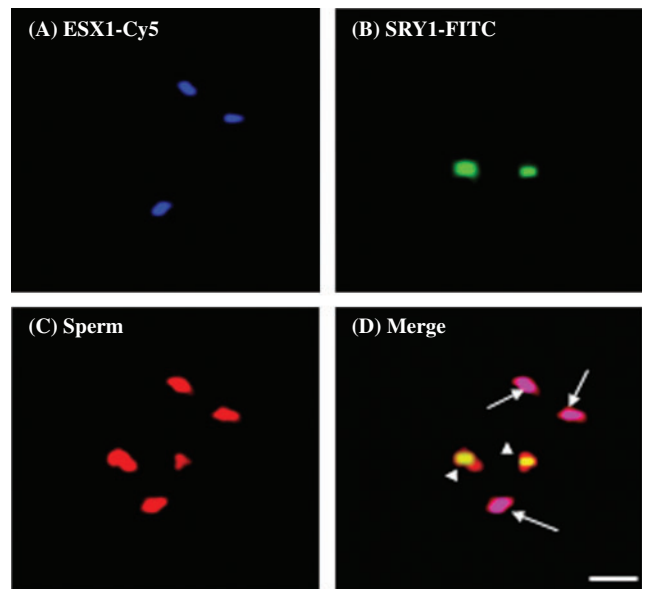


Fig. 5. Distinct localisation of extra-embryonic tissue-spermatogenesis-homeobox gene 1 (ESX1) and sex-determining region Y chromosome (SRY) proteins in mouse sperm. The blue colour and green fluorescence indicate the locations of ESX1 and SRY, respectively (A,B); the red colour represents sperm counterstained with propidium iodine (C). (D) A merged diagram showing purple (arrows) and yellow (arrowheads) colours indicates the location of ESX1 and SRY in sperm nuclei respectively. Scale bar = 5 μ m.

Table 1. Distribution of X and Y chromosome-bearing sperm after separation by swim-up proceduresValues are means \pm s.e.m.

Treatment	X chromosome-bearing sperm (%)	Y chromosome-bearing sperm (%)	<i>P</i>
None (<i>n</i> = 4)	49.9 \pm 2.0	50.1 \pm 2.0	NS
Swim-up (<i>n</i> = 4)	46.7 \pm 3.3	53.3 \pm 3.3	NS

Y chromosome sperm population from the X chromosome sperm (Andersen and Byskov 1997; Seidel and Johnson 1999). Approximately 10 000 sperm were examined using confocal laser microscopy. Over 95% of sperm examined gave positive signals for each sample collection. The overall ratio of X chromosome sperm to Y chromosome sperm without swim-up separation was 49.9 : 50.1. The ratio of X chromosome sperm to Y chromosome sperm separated by the swim-up technique was 46.7 : 53.3 (Table 1).

Discussion

Sperm preselection on the basis of preferred sex before conception has been proposed to avoid the tragic or deadly sex-linked hereditary disorders in humans, to conserve endangered species and to mass produce single-sexed laboratory animals (Jafar and Flint 1996; Reubinoff and Schenker 1996; Howes *et al.* 1997; Hendriksen 1999; Seidel and Johnson 1999). Previous studies have focused mainly on the physical and chemical distinguishing characteristics of sperm. However, there has not been any irrefutable evidence showing any particular differences between sperm bearing the X or Y chromosome (Han *et al.* 1993; Howes *et al.* 1997). Nevertheless, immunological approaches have been used to differentiate sperm using the monoclonal H-Y antibody (Epstein *et al.* 1980; Ali *et al.* 1990; Andersen and Byskov 1997; Gardon *et al.* 2004). This male-specific H-Y antigen, which is only expressed in male cells, has been identified by various techniques, including two-dimensional SDS-PAGE (Blecher *et al.* 1999). In 1990, Ali *et al.* identified and separated Y chromosome-bearing sperm from X chromosome-bearing sperm using monoclonal H-Y antibodies (Ali *et al.* 1990). Their results showed that specific H-Y antibody could bind to approximately half the sperm. However, they were not able to alter the sex ratio of offspring after treatment of sperm with monoclonal H-Y antibodies. Similarly, the goal of the present study was to elucidate a candidate marker specifically for X chromosome-bearing sperm. In the present study, we have identified a new candidate protein marker, namely ESX1, expressed in the later stage of spermatogenesis and exclusively expressed in the population of X chromosome-bearing sperm (Fig. 5). The present study provides the first example of a truly X chromosome-bearing sperm-specific protein marker to differentiate X and Y

chromosome-bearing sperm. Ultimately, our antiserum could be used to separate X from Y chromosome-bearing sperm.

Previous reports have demonstrated that most of the genes on sex chromosomes are not translated during the later stages of spermatogenesis (Salas-Cortes *et al.* 1999; Shinohara *et al.* 2000). However, the proteins expressed in post-meiotic germ cells, especially in spermatids, apparently play essential roles in spermatogenesis and even in differentiating X and Y chromosome-bearing sperm (Koopman *et al.* 1990; Peterson *et al.* 1992; Odorisio *et al.* 1996). To investigate the spatial expression of ESX1 in testicular germ cells, we separated the spermatogenic cell populations from testes by centrifugal elutriation. By separating the different germ cell populations, it was possible to verify the differential localisation of ESX1 in testicular germ cell development (Mays-Hoopers *et al.* 1995). After analysing the expression of ESX1 in the testicular germ cells and cryptorchid testis by Western blot and immunofluorescent analyses, we concluded that ESX1 started to appear in the spermatids at 3 weeks of age. No ESX1 was expressed in spermatogonia and spermatocytes. However, two previous studies demonstrated that the expression of *Esx1* mRNA in spermatogonia/preleptotene spermatocytes was abundant (Branford *et al.* 1997; Li *et al.* 1997). No explicit explanation is currently available for the contradictory results. Nevertheless, there is an agreement of the absence of *Esx1* mRNA expression in spermatocytes; in turn, ESX1 may be a result of meiotic sex chromosome inactivation (Branford *et al.* 1997; Li *et al.* 1997). The other intriguing finding of the present study was that ESX1, unlike *Esx1* mRNA, which was found in both X and Y chromosome-bearing spermatids, existed only in half the sperm population.

The other interesting finding of the present study is the demonstration of the presence of SRY protein in the adult testis (Fig. 4). Previous reports indicated that *Sry* transcripts are abundantly expressed in meiotic and post-meiotic cells, yet they are translationally inactive because they are circular (Capel *et al.* 1993; Rossi *et al.* 1993). However, other groups have indicated that SRY protein is present mainly in the nuclei of fetal and adult gonadal tissue, including germ cells (Cohen *et al.* 1994; Salas-Cortes *et al.* 1999). These contradictory observations require further investigation.

There are many methods used to identify and separate X and Y chromosome-bearing sperm, including centrifugation, swim-up and sedimentation (Vidal *et al.* 1993; Risopatron

et al. 1996). The only reliable technique so far available to separate X and Y chromosome-bearing sperm was using flow cytometry with fluorochrome dye according to the different DNA content of the X and Y chromosomes (Johnson *et al.* 1989; Johnson 2000). The fluorescent *in situ* hybridisation method was the most common method to determine the efficiency of the separation techniques. However, this method needs specific DNA probes on the X or Y chromosome conjugated with fluorescence. It is a time-consuming and expensive method (Han *et al.* 1993; Andersen and Byskov 1997; Johnson and Welch 1999a, 1999b). In the present study, the immunostaining method using ESX1 antiserum proved to be a powerful technique in identifying X chromosome-bearing sperm. Although our antiserum is very good for the identification of X chromosome-bearing sperm, it appears less likely that our antiserum would be used for sperm separation because its epitope is within the nucleus instead of on the outer membrane of the sperm.

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