Summary. Mammalian spermatozoa lose plasma membrane cholesterol during maturation in the epididymis and during capacitation in the female reproductive tract. While cholesterol acceptors such as high-density lipoproteins (HDL) and apolipoproteins A-I (apoA-I) and J (Apo J) have been found in male and female reproductive tracts, transporters that mediate cholesterol efflux from plasma membranes of spermatozoa to acceptors are not well defined. Candidates include members of the ATP-binding cassette (ABC) transporter superfamily including ABCA1, ABCA7, ABCA17, and ABCG1. In this study, we utilize immunocytochemistry on sections of adult mouse testis and epididymis and RT-PCR on isolated germ cells. The data reveal that ABCA17 is expressed by steps 12-16 elongated spermatids in the mouse in testis and by spermatozoa in the lumen of the epididymis where ABCA17 localizes to the sperm head and tail midpiece. It also localizes on these areas of mouse sperm isolated from the epididymis. Moreover, ABCA17 antibody interferes with cholesterol efflux from spermatozoa to lipid acceptors apoA-I. Taken together, these results suggest that ABCA17 plays an important role in the process of sterol efflux which renders spermatozoa capable of fertilizing an oocyte.

Key words: ATP-binding cassette transporters, ABCA17, Cholesterol efflux, Mouse testis, Sperm

Introduction

Maturation and capacitation of mammalian sperm are required steps for fertilization. While the former entails a multitude of events that are coordinated by the epithelial cells lining the epididymis (Robaire and Hermo, 1988; Turner and Bomgardner, 2002; Robaire and Henderson, 2006; Hermo et al., 2010), the latter is a functional maturation step involving changes in both the sperm’s ability to undergo the acrosome reaction and the acquisition of a hyperactivated pattern of motility occurring in the uterus and oviduct (Davis, 1981; Visconti et al., 1995). Although poorly understood, a decrease in sperm cholesterol levels is essential, and this change begins at the time of sperm maturation and culminates during capacitation (Davis, 1981; Benoff, 1993; Visconti et al., 1995; Cross, 1998; Travis and Kopf, 2002). It is mediated by lipid-binding proteins and the ATP-binding cassette (ABC) transporters present in the epididymis, uterus and oviduct (Martinez and Morros, 1996; Borntick et al., 2000).

In the epididymis, the distribution of apolipoprotein AI (apoAI) and apolipoprotein J (apoJ), two major lipid-binding proteins and constituents of high density lipoproteins (HDL) has been defined (Argarves and Morales, 2004). Both apoAI and apoJ are secreted by the epithelial epididymal principal cells (Law et al., 1997) into the lumen of the duct where they interact with sperm plasma membranes (Hermo et al., 1991; Sylvester et al., 1991; Law et al., 1997). After a transient interaction with the sperm surface, both apoAI and apoJ dissociate and are internalized by the epididymal epithelial cells via the endocytotic receptors, megalin (low density lipoprotein 2, LRPG2) and cubilin (Hermo et al., 1991; Morales et al., 1996; Van Praet et al., 2003). ApoAI and apoJ are also expressed in the female
reproductive tract by the uterine and oviduct epithelial cells (Jaspard et al., 1996), and incubation of ejaculated sperm with oviductal fluid causes a transfer of cholesterol from sperm to apoAI-containing HDL present in this fluid (Martinez and Morros, 1996). Although the mechanism by which cholesterol and phospholipids efflux from the sperm plasma membrane to lipid acceptors is poorly known, ATP-binding cassette (ABC) transporters represent *bona fide* candidates in this process (Selva et al., 2004).

The ABC transporters form one of the largest protein families. Currently the human genome codes for 49 distinct ABC transporters and the mouse genome has 53. These are divided into 7 subfamilies (ABCA-ABCG) based on amino acid sequence similarities and phylogeny (Klein et al., 1999; Biemans-Oldehinkel et al., 2006; Davidson and Maloney, 2007; Dawson et al., 2007; Hollenstein et al., 2007a; Velamakanni et al., 2007). ABC transporters include the P-glycoprotein (P-gp) and multidrug resistance proteins (Klein et al., 1999; Hollenstein et al., 2007b; Potocnik et al., 2008). ABC transporters are recognized by consensus ATP-binding domains also known as nucleotide-binding domains (NBDs) which have several conserved sequence motifs all with specific functions (Jones and George, 2004; Oldham et al., 2008). NBDs contain characteristic motifs Walker A and Walker B separated by about 90-120 amino acids. ABC proteins also contain an additional element the signature C motif located just upstream of the Walker B site (Gottesman et al., 2002). In addition, ABCs contain 2 transmembrane domains (TMDs) composed of 6-11 membrane-spanning α-helices. ABC transporters are involved in transport of substances such as sugars, sterols, amino acids, bile acids, glycerans, cholesterol, phospholipids, peptides, proteins, toxins, antibiotics and xenobiotics (Higgins, 1992; Várádi and Sarkadi, 2003). They bind and hydrolyze ATP and use this energy to move substrates into and out of cells and organelles, or to flip molecules from the inner to the outer leaflet of the membrane (Higgins, 1992; Dean et al., 2001). Some ABC transporters are not directly involved in moving substrates but appear to be part of regulated ion channels such as CFTR. Others function in multi-drug extrusion of toxic substances, which can lead to resistance of cancer cells against drugs used in chemotherapy (Gottesman et al., 2002). A large percentage of ABC genes cause diverse human genetic diseases and at least 17 ABC genes have been linked to disorders displaying Mendelian inheritance including some involved with cholesterol and lipid transport (Klein et al., 1999).

In the testis, ABCA1 and ABCA7 are present in Sertoli cells (Morales et al., 2008), and ABCA1-deficiency leads to a 21% reduction in the ability of mice to sire offspring (Selva et al., 2004). ABCA1 and ABCA7 also localize to the heads and tails of spermatozoa, where they play a role in cholesterol efflux (Morales et al., 2008). An Abca3-like gene in the sea urchin genome, suAbca, is expressed in sperm and may be involved in shedding of cholesterol during sperm head maturation (Mengerink and Vacquier, 2002). ABCG1 has also been shown to be expressed on the tails of sperm and to be involved in cholesterol efflux (Morales et al., 2008). While ABCG4 has been reported to mediate cholesterol efflux (Wang et al., 2004), and to be expressed in the testis (Koshiba et al., 2008), Morales et al. (2008) did not detect ABCG4 transcript or protein in germ cells. In addition, ABCC9/SUR2 and Kir6.2 (KCNJ11) are expressed in the epididymis and luminal sperm (Lybaert et al., 2008), and Bcrp1/ABCG2 in spermatogonia and spermatids (Lasalle et al., 2004; Scharenberg et al., 2009). However, these transporters are not directly involved in cholesterol efflux.

Recent findings show that accumulation of cholesteryl esters in LXR−/− mice is associated with a specific loss of ABCA1 and an increase in apoptosis of epithelial cells of the proximal caput epididymidis (Ouvrier et al., 2009). Whether or not the described pro-apoptotic effects are directly related to ABCA1 deficiency is not known. LXR is also a positive regulator of the expression of ABCG1 (Beyea et al., 2007). In addition to the above transporters that appear to play significant roles in sperm, a novel testis-specific transporter termed ABCA17 has also been cloned and it has been suggested that this protein is involved in efflux of esterified neutral lipids, such as cholesteryl esters, fatty acid esters and triacylglycerols (Ban et al., 2005).

To further unfold the specific role of ABCA17 in the testis and epididymis and characterize its cellular distribution, the present study pursued three objectives: 1) To investigate the cellular distribution of this protein both in vivo and in isolated sperm epididymal samples by light microscope immunocytochemistry using a novel zinc fixation method and fluorescence imaging, as well as by traditional fixatives; 2) To determine the expression of ABCA17 by northern blot analysis in the adult mouse testis and by RT-PCR on isolated germ cell populations of different postnatal ages; 3) To test the hypothesis that ABCA17 is involved in cholesterol efflux in mouse sperm. Taken together, the results reveal that germ cells express ABCA17 and that it plays a role in efflux of cholesterol from sperm.

**Materials and methods**

**Antibodies**

Polyclonal ABCA17 antibody was raised in rabbit against a synthetic peptide corresponding to 19 C-terminal amino acid residues (SSPTPKLPSPPPSSPILL) of mouse ABCA17, which show no similarity to other members of the ABCA subfamily. The antibody was purified using affinity chromatography MabTrap Protein G (Amersham Biosciences). Its specificity and characterization was published previously by Ban et al. (Ban et al., 2005) Rabbit polyclonal antibody to ABCG1...
ABC17 expression in mouse testis

was purchased from Novus Biologicals (Littleton, CO) and its specificity and characterization were published previously by Morales et al. (2008).

Immunocytochemistry

Three retired male breeder mice (CD-1 strain, Charles River Laboratories, Montreal, QC, Canada) were anesthetised and their testis, efferent ducts and epididymis were fixed by cardiac perfusion with Bouin’s solution or a zinc fixative solution (BD Biosciences, Mississauga, ON, Canada) (Hermo et al., 2008). After 10 minutes of perfusion, the tissues were removed and placed in fresh fixative overnight. On the following day, the tissues were dehydrated and embedded in paraffin wax, after which they were sectioned at 5 µm thickness. In the case of the Bouin’s fixed tissues, the sections were dewaxed in Citrisolv, rehydrated in a reverse ethanol gradient and washed in distilled water, followed by 50 mM Tris-buffered saline (TBS), pH 7.4. Antigen retrieval was carried out by heating sections for a total of 15 min at 85-95°C in 10 mM sodium citrate buffer (pH 6.0) containing 0.1% Tween 20 (Sigma-Aldrich Canada, Oakville, ON, Canada). Sections were then washed with 50 mM Tris, 0.3 M NaCl, 0.1% Tween 20, pH 7.4, (TBST) and incubated for 30 min at room temperature with anti-ABC17 antibody diluted in 0.05 M Tris-HCl with 0.1% BSA, pH 7.4. Sections were washed again in TBST and incubated for 30 min with a goat anti-rabbit HRP labeled polymer (DakoCytomation, Carpinteria, CA). After washing in TBST, the slides were incubated in a DAB substrate-chromogen solution (DakoCytomation) for 30 min, rinsed in TBS and counterstained with a 0.1% methylene blue and 0.1% thionin solution. The slides were then dehydrated and mounted with Permount.

In the case of the zinc fixed tissues, sections were dewaxed with hexane, rehydrated in a reverse ethanol gradient and washed with TBS followed by TBST. The sections were blocked for 20 min with 2% skim milk in TBS and incubated for 3 hours at room temperature with the rabbit antibody diluted 1:100 in 0.05 M Tris-HCl with 0.1% BSA, pH 7.4. Sections were washed in TBST, blocked again with skim milk and incubated for 30 min with a goat anti-rabbit Alexafluor 594-labeled goat anti-rabbit IgG antibody (Invitrogen Canada) diluted 1:500 in TBST. Samples were washed in TBST followed by TBS, and counterstained for 1-3 min at room temperature with 300 nM 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen Canada) in TBS. Samples were rinsed in TBS and coverslips were mounted using Prolong Gold antifade reagent (Invitrogen Canada). The tissues were examined and photographed on a Zeiss Axioskop 2 motorized light microscope equipped with variable intensity FluorArc epifluorescence mercury lighting and AxioCam HR color digital camera (Carl Zeiss Canada; Montreal, QC, Canada). Control sections consisted of elimination of the primary antibody and use of non-immune rabbit serum.

Spermatozoa immunostaining

Three retired male breeder mice (CD-1 strain, Charles River Laboratories, Montreal, QC, Canada) were anesthetised with sodium pentobarbital. The caput epididymis of each animal was removed and placed in Krebs Ringer bicarbonate medium buffered with HEPES, and minced with a razor blade. The spermatozoa-containing supernatant was collected and centrifuged at 800xg, at room temperature for 5 min. The pelleted spermatozoa were resuspended in 2 ml PBS. Approximately 100 µl of the suspension was deposited onto a glass slide and dried at room temperature. The slides were rehydrated in PBS during 5 min and fixed with 3.7% formaldehyde for 10 min at room temperature. The slides were washed three times in PBS and blocked with 3% goat serum and 2% horse serum for 30 min at room temperature. The slides were incubated with ABC17 antibody for 60 min at room temperature and washed three times in PBS. Subsequently, the slides were incubated with FITC-conjugated secondary antibodies for 60 min at room temperature followed by three washes in PBS. The nuclei were counterstained with Hoechst 33342 (Molecular Probe, Eugene Oregon). Control slides were reacted with non-immune rabbit IgG. The slides were examined with a LSM 510 confocal microscope (Carl Zeiss, Montreal, QC).

Germ cell preparation for RNA isolation

The separation of germ cells from the testis was done following a procedure established in our lab (Petrie and Morales, 1992). Three retired male breeder mice (CD-1 strain, Charles River Laboratories, Montreal, QC, Canada) were anesthetised with sodium pentobarbital and their testes removed by an abdominal incision. The testes were washed with Hank’s Balanced Salt Solution (HBSS), decapsulated and lightly minced. The tissue was then suspended in 10 ml of HBSS containing 0.4 mg collagenase, 0.6 mg deoxyribonuclease I (DNase I), 6 mM sodium pyruvate and 0.2 mM lactate. The suspension was incubated at 30°C for 10 min followed by an additional 15 min incubation period with 18 mg trypsin, with intermittent agitation during the entire procedure. The supernatant containing the germ cells was then spun down at 700xg for 5 min and the cells resuspended in 10 ml HBSS containing 1.8 mg trypsin inhibitor and 0.6 mg DNase. The cell suspension was then spun at 700xg for 5 min, resuspended in 1% bovine serum albumin-HBSS (BSA-HBSS), and sequentially filtered through 80 µm and 35 µm mesh to remove cellular clumps and debris. The cell suspension was finally layered into a statup chamber and left to sediment for 2 h in a 200 ml linear 1-4% BSA density gradient with HBSS. Twenty 10 ml fractions were collected and
each was examined by contrast-phase microscopy for identification and assessment of germ cell type and percentage per fraction. Only those fractions containing spermatogonia/spermatocytes, round spermatids, elongated spermatids, cytoplasm/residual bodies in purities of at least 70% were pooled and used to isolate their respective content of RNA.

RT-PCR

Four different germ cell fractions (pachytene spermatocytes, round spermatids, elongated spermatids and residual bodies/cytoplasts) were collected and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Mississauga, ON), according to manufacturer’s specifications. A fraction of 2 µg of total RNA was used to synthesize cDNA. RT-PCR reaction was performed using Omniscript RT kit (Qiagen, Mississauga, ON). Same amounts of cDNA were used to amplify ABC17, the loading control was β-actin. ABC17 primers were designed based on mouse ABC17 cDNA sequence accession number NM_001031621. The ABC17 forward primer was 5’-CAGAGTGCGCTGTGTTCAGGA and the ABC17 reverse primer was 5’-CCTCACACTGCCTATGG corresponding to bases 993-1012 and 1300-1319, respectively. The predicted amplification size was 427 bp. PCR cycling conditions were 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension for 5 min at 72°C.

Northern blot analysis

Total RNA was extracted from the testes of mice at postnatal days 5 (n=3), 20 (n=3), and 45 (n=3) and from different germ cell fractions using a RNeasy Mini Kit (Qiagen, Mississauga, ON). RNA blot analysis was carried out using a 32P-labelled DNA fragment, which corresponded to nucleotides 2479 to 3342. The hybridization was done under high-stringency conditions as described previously (Ban et al., 2005). Briefly, total RNA (20 µg) was denatured and electrophoresed in 1% (w/v) agarose gel, blotted on a nylon membrane, and hybridized with the ABC17 cDNA probe. The membrane was exposed to X-ray film with an intensifying screen at -80°C for 4 days.

Immunoblot analysis

Male CD-1 mice at postnatal days 5 (n=3), 20 (n=3), and 45 (n=3) were anesthetised with sodium pentobarbital. Their testes were removed and placed in Dulbecco’s Modified Eagle Medium (Invitrogen Corporation, Burlington, ON) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche, Palo Alto, CA) and minced using a razor blade and pelleted. The pelleted tissue was resuspended in 100 µl of 1.0% NP40, 154 mM NaCl, 0.4mM Tris pH 8.0 containing protease inhibitors (Roche). After 30 min incubation, the lysate was centrifuged at 10,000xg, 4°C for 10 min. The protein concentration of the supernatant was determined by BioRad Protein Assay (BioRad, Mississauga, ON). Aliquots containing 20 µg total protein were subjected to SDS-PAGE under reducing conditions and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Detection was achieved using the ECL+ Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) and Kodak BioMax Light Film (Kodak, Montreal, QC). Lysates from different germ cell fractions were also subjected to SDS-PAGE, transferred to Hybond-ECL nitrocellulose membranes and stained with the ABC17 antibody as described above.

Spermatozoa cholesterol efflux assays

The caput, corpus and cauda epididymides were isolated from euthanized CD1 mice (n=3). The tissue was chopped using a razor blade and transferred to 5 ml of Krebs Ringer bicarbonate medium buffered with HEPES (KRB-HEPES) and kept at 37°C in a water bath for 10 min. The released spermatozoa were pelleted by centrifugation at 500xg, for 10 min at 25°C. The pellets were resuspended in 2 ml of KRB-HEPES (37°C) at a concentration of 1x10⁷ spermatozoa/ml. Subsequently, 100 µl of the cell suspension was mixed with 100 µl of 50 µg/ml human apoAI (Sigma) (in KRB-HEPES) with either: 1) rabbit IgG plus 0.5% NP40; 2) 0.5% NP40; 3) ABCG1 (10 µg IgG/ml) plus 0.5% NP40; 4) ABCA17 (10 µg IgG/ml) plus 0.5% NP40; 5) ABCA17 (10 µg IgG/ml) plus ABCG1 (5 µg IgG/ml) plus 0.5% NP40; and incubated at 37°C and 5% CO₂ for 1 h. After 1 h incubation, spermatozoa were pelleted by centrifugation at 500xg for 10 min at 25°C. The supernatant was then transferred to a new tube and the spermatozoal pellet was washed once with KRB and then resuspended in 140 µl of PBS. Aliquots (50 µl) of the spermatozoal suspension and supernatant were analyzed for cholesterol using an Amplex Red Cholesterol Kit (Molecular Probes, Eugene, OR) and a PerkinElmer Life Sciences Microplate Reader (PerkinElmer Instruments, Wellesley, MA). Statistical analysis was performed using the t-test.

Results

ABCA17 localization and expression in mouse testis

Immunoperoxidase staining of adult mouse testis sections fixed in Bouin’s and reacted with anti-ABCA17 antibody revealed a weak staining of spermatogonia and early spermatocytes and a moderate staining of the cytoplasm of pachytene spermatocytes (Figs. 1A,B). While ABCA17 was not detected in early spermatids, a reaction was noted in the heads and tails of step 12-16 spermatids (stages XII-VIII of the cycle of the seminiferous epithelium) (Fig. 1A,B). Control sections
incubated with non-immune rabbit IgG were completely devoid of staining (Fig. 1C,D).

Spermatozoa isolated from the caput epididymidis and reacted with anti-ABCA17 antibody revealed ABCA17 expression on the dorsal aspect of the sperm heads and on the midpiece of sperm tails (Fig. 1E). By contrast, no reaction was detected in spermatozoa incubated with non-immune rabbit IgG (Fig. 1F).

Immunofluorescent staining of testicular (Fig. 2A) and epididymal (Fig. 2B,C) sections treated with zinc fixative revealed a strong reaction on the heads and tails of late spermatids and spermatozoa. Spermatogonia, and pachytene spermatocytes were unreactive. Control sections incubated with non-immune rabbit IgG were

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**Fig. 1.** Immunolocalization of ABCA17 in mouse seminiferous tubules fixed with Bouin’s and in spermatozoa isolated from caput epididymidis. **A**, reveals anti-ABCA17 immunoperoxidase staining of a seminiferous tubule at stage II of the cycle. Arrows in **A** point to staining of spermatogonia, pachytene spermatocytes and step 16 spermatids. **B**, shows an immunoperoxidase staining with anti-ABCA17 of a seminiferous tubule at stage VII of the cycle. Arrows in **B** point to staining of spermatogonia, pachytene spermatocytes and step 13 spermatids (located at the luminal border). **C** and **D** are controls IgG immunocytochemical staining of seminiferous tubule at stages II and VII. Bar in panel **D** equals 25 µm and applies to panels **A-C**. **E**, shows a confocal microscope field of spermatozoa isolated from caput epididymidis immunolabeled with ABCA17 antibody and detected with FITC-conjugated anti-IgG (green). Nuclei were stained with Hoechst 33342 (blue). **F**, is a control IgG staining. Bar in panel **F** equals 10 µm and applies to **E**.
ABCA17 expression in adult mouse testis

ABCA17 transcripts were detected by Northern blot analysis in total testicular RNA isolated from mice at postnatal day 5, 20 and 45. Expression of ABCA17 transcripts (5.3 Kb) in the testis was observed at low levels at day 5, correlating with the appearance of spermatogonia. At postnatal day 20, ABCA17 mRNA expression increased, corresponding with the developmental appearance of pachytene spermatocytes. The level of ABCA17 transcripts further increased in the testis of 45 day old mice, consistent with the presence of both early and elongated spermatids (Fig. 3A).

ABCA17 protein was detected in Western blots of testes at postnatal day 5, 20 and 45. Consistent with findings from Northern blot analysis, relatively low levels of ABCA17 were detected in testicular extracts at postnatal day 5 old mice. However, the level of ABCA17 increased in testis at postnatal day 25 and 45 (Fig. 3B).

Expression of ABCA17 in isolated testicular cells

Spermatogonia, spermatocyte, round spermatids and elongated spermatids were isolated at 70-90% purity by

Fig. 2. Immunostaining of ABCA17 in mouse testicular A and epididymal B-D sections preserved with zinc fixative. A. ABCA17 antibody reacts specifically with elongated spermatids (stage VI of the cycle). B, C. ABCA17 antibody also produces a strong immunofluorescence staining of epididymal luminal spermatozoa (B, caput; C, cauda). D, is a control cauda section incubated with non-immune rabbit IgG. Bar in panel D equals 30 µm and applies to all panels.
staput velocity sedimentation on a 1-4% BSA gradient in HBSS. The spermatogonial/spermatocyte fraction (F1; 80% purity) was mainly contaminated with round spermatids. The round spermatid fraction (F2; 90% purity) contained 10% of elongated spermatids. The elongated spermatid fraction (F3; 80% purity) was chiefly contaminated with round spermatids and cytoplasm and the cytoplasm/residual bodies/spermatozoal fraction (F4; 90% purity) was contaminated with elongated spermatids. RT-PCR was performed on total RNA isolated from each of the four fractions. The analysis revealed a strong expression of ABCA17 mRNA expression in fraction 1 and 2 and a slight decrease in fraction 3 and 4 (Fig. 4A). Northern blot analysis demonstrated decreasing levels of ABCA17 transcripts in fraction 3 and 4 (Fig. 4B). Immuno blotting of the cell lysates showed low level of expression of ABCA17 in fraction 2 and high levels in fractions 1, 3 and 4. Control β-actin remained unchanged (Fig. 4C). The plot (Fig. 4D) illustrates the quantitative levels of ABCA17 mRNA and protein relative to β-actin.

Involvement of specific ABC transporters in spermatozoa cholesterol efflux

Cholesterol efflux analysis was performed on isolated spermatozoa obtained from the caput, corpus and cauda epididymidis incubated in the presence or absence of the anti-ABCA17 antibody (10µg IgG/ml) according to the procedure of Morales et al. (2008). Because the antibody was raised against an internal region of ABCA17, the spermatozoal membrane was permeabilized with a mild treatment of NP40. The ABCA17 antibody in the presence of NP40 produced a 40% inhibition of cholesterol efflux to apoA-1 in spermatozoa isolated from the corpus epididymidis. The inhibition was statistically significant (p<0.05) (Fig. 5). This inhibition was further increased when ABCA17 was incubated in the presence of 5 µg IgG/ml of ABCG1. A decrease was also observed in the cauda epididymidis, however, the data was not statistically significant. NP40 alone did not cause any efflux towards the lipid free apoAI. Consistent with the observation that the ABC antibodies reduced cholesterol efflux was the finding that ABC antibody treatment caused an increase in cell-associated cholesterol (7-15% greater) as compared to spermatozoa treated with control IgG (data not shown).

Discussion

Efflux of plasma membrane cholesterol from mammalian sperm occurs during sperm maturation in the epididymis and during capacitation in the uterus and oviduct (Davis, 1981; Visconti et al., 1995). The loss of cholesterol is essential for the sperm acrosome reaction and hyperactivated motility (Davis, 1981; Visconti et al., 1995; Martinez and Morros, 1996; Jones, 1998). In fact, a variety of events leading up to acrosome reaction are mediated by the loss of cholesterol, such as changes in intracellular ion concentrations, increase in intracellular pH, lipid raft migration to the sperm head and initiation of protein phosphorylation-based signaling cascades (Cross and Razy-Faulkner, 1997; Visconti et al., 1999; Shadan et al., 2004). The efflux of sterols from spermatozoa plasma membranes requires the presence of lipid acceptor molecules (Davis, 1981; Benoff, 1993; Cross, 1998; Travis and Kopf, 2002), and several publications have documented the existence of lipid acceptor molecules such as high-density lipoproteins (HDL) and apolipoproteins A1 and J in both the male and female reproductive tracts (Hermo et al., 1991; Sylvester et al., 1991; Jaspard et al., 1996; Martinez and Morros, 1996; Law et al., 1997; Argraves and Morales, 2004).

However, an essential step in this process is the presence

ABC17 expression in mouse testis

Fig. 3. Developmental expression of ABCA17. A, is a Northern blot analysis of testicular RNA and from postnatal 5, 20 and 45 day old mice. The blots are total testicular RNA hybridized with an ABCA17 probe showing low expression of ABCA17 transcripts (5.3 Kb) in testis from postnatal day 5 mice. The level of transcripts increased at postnatal day 20 and 45 (left panel). The right panel is an internal control of the same blot reacted with a GAPDH probe showing unchanged levels of transcripts. B, is an immunoblot of testicular proteins isolated from postnatal 5, 20 and 45 day old mice reacted with anti-ABCA17 antibody (left panel). Low level of ABCA17 was observed at postnatal day 5. The levels of ABCA17 protein increased at postnatal day 25 and 45. The right panel is a control of the same blot reacted with a GAPDH antibody.
of integral proteins capable of transporting sterols from the plasma membrane to the lipid acceptor molecules (Klein et al., 1999). In a past study, our laboratory identified three members of the ATP-binding cassette (ABC) transporter superfamily, ABCA1, ABCA7, and ABCG1 in the sperm plasma membrane (Morales et al., 2008). They were reported to be implicated in the transport of cholesterol to apoA1 and albumin. In fact, antibodies to all three of these transporters affected cholesterol efflux from the sperm to lipid acceptors apoA1 and albumin and inhibited in vitro fertilization.

Fig. 5. Effect of ABCA17 antibody treatment on spermatozoa cholesterol efflux. The graph shows levels of spermatozoa cholesterol efflux to apoA-I in the absence or presence of NP40 and antibodies to ABCA17 and ABCG1. In control reactions, spermatozoa were incubated in presence or absence of IgG or NP40. Statistical analysis was performed by t-test. Asterisks indicate that when compared to the three controls the difference is significant (p<0.05).

Fig. 4. Expression of ABCA17 in isolated testicular cells. A, is a RT-PCR analysis of ABCA17 mRNA expression in adult mouse testis. The RT-PCR was performed in total RNA isolated from germ cells. Fraction 1 (SP) contained spermatogonia and spermatocytes, fraction 2 (RS) round spermatids, fraction 3 (EP) elongated spermatids and cytoplasm, and fraction 4 (RB) cytoplasts, residual bodies and spermatozoa. The analysis revealed a strong expression of ABCA17 mRNA in fractions 1 and 2 and a gradual reduction in fractions 3 and 4 (left panel). Control β-actin remained unchanged (lanes 1-4, right). B, is Northen blot analysis of ABCA17 mRNA expression in adult mouse testis. The blot was performed on total RNA isolated from germ cells as depicted in A, revealing high levels of ABCA17 mRNA in fractions 1 and 2 and decreasing levels of ABCA17 transcripts in fractions 3 and 4 (left panel). The right panel is an internal β-actin control. C, is an immunoblot of testicular proteins from isolated germ cells reacted with anti-ABCA17 antibody (left panel). The immunoblot revealed low expression of ABCA17 in fraction 2 and high level of expression in fractions 1, 3 and 4 (left panel). Control β-actin remained unchanged (right panel). D, illustrates the quantitative levels of ABCA17 mRNA (RT-PCR and Northern blot analysis) and protein (immunoblot analysis) relative to β-actin.
ABC A17 expression in mouse testis

(Morales et al., 2008).

In a recent study, the molecular cloning of the full length cDNA of a novel mouse ATP-binding cassette transporter, ABC A17, which belongs to the A subfamily has been identified (Ban et al., 2005). Metabolic labeling analysis showed that intracellular esterified lipids, including cholesteryl esters were significantly decreased in HEK293 cells stably expressing ABC A17 compared to untransfected cells. Northern blot analysis and quantitative real time PCR revealed that ABC A17 mRNA was expressed exclusively in the testis. While in situ hybridization showed the presence of transcripts in spermatocytes, immunofluorescence with an anti-ABC A17 antibody localized the protein in the anterior head of elongated spermatids and sperm (Ban et al., 2005).

The findings presented herein indicate that ABC A17 transcripts are expressed in all germinal cells. Indeed, both RT-PCR and Northern blot analysis of spermatogonia, spermatocytes, round spermatids and elongated spermatids isolated by stepwise velocity sedimentation showed strong expression of ABC A17 mRNA. Immunoperoxidase staining with ABC A17 antibody confirmed that, with the exception of round spermatids, protein is present in spermatogonia, spermatocytes and elongated spermatids. The observation of ABC A17 transcripts and protein in spermatogonia has not been reported before and suggests that ABC A17 may play an important role during spermatogonial differentiation.

The immunoperoxidase staining technique used in this study is based on polymer intensification of reaction product which allows detection of very low levels of antigen. This contrasts to the immunofluorescent staining procedure which is essentially one antigen-to-one antibody reaction. The finding that only elongated spermatids were reactive by immunofluorescence is consistent with the likelihood that these cells contain much higher amounts of translated protein than do spermatocytes or spermatogonia. The presence of high levels of ABC A17 transcripts in round spermatids and high expression of ABC A17 protein in elongated spermatids and spermatooza raises the possibility that the ABC A17 mRNA is posttranscriptionally regulated during spermiogenesis. Indeed, a number of testis-specific mRNAs are maximally transcribed in round spermatids at mid steps of spermiogenesis, although the peak amounts of proteins are synthesized days later in late elongated spermatids (Kleene et al., 1984; Hecht, 1989; El-Alfy et al., 1999; Morales et al., 2002). Therefore, it is entirely possible that the synthesis of mouse ABC A17 protein in late spermatids occurs long after the cessation of transcription in round spermatids.

Using Northern blot analysis, the present study showed that ABC A17 mRNA was expressed in testis at postnatal day 5, with expression gradually increasing at postnatal day 25 and postnatal day 45. The period of maximal ABC A17 mRNA expression corresponded to the time point when germ cells were fully differentiated. Immunoblotting of testicular lysates reacted with ABC A17 antibody showed low levels of protein expression at postnatal day 5 correlating with the presence of spermatogonia. At postnatal day 20, ABC A17 protein expression increased substantially, corresponding to the developmental appearance of pachytene spermatocytes. The level of protein further increased in the testis at postnatal day 45, consistent with the presence of elongated spermatids and spermatooza. Thus, these results validated our observations on ABC A17 expression obtained on isolated germ cells.

Unlike ABC A1, ABC A7 and ABC G1 reported before by us in the sperm plasma membrane, ABC A17 is the first testis-specific transporter with a distinct topographical distribution. In addition to its presence in the mouse testis noted in the present study, ABC A17 has been found in rat testis (Ban et al., 2005) but its subcellular localization is presently unknown. Our studies show that ABC A17 is expressed differently in two important regions of the spermatooza, the posterior region of the head and the mid piece of the tail. This unique distribution of ABC A17 may correlate with regionally distinct responses to lipid acceptor treatments such as the induction of acrosome reaction and motility. It is interesting to note that treatment of spermatooza with the lipid acceptor albumin is known to alter the lipid composition of the plasma membrane with respect to distinct structural and functional regions of the sperm (Wolf et al., 1986). These changes are suspected to play a major role in the induction of acrosome reaction and hyperactivation of flagellar activity (Wolf et al., 1986).

In humans, the rodent Abca17 gene is a ubiquitously expressed pseudogene (ABC A17P). Interestingly, ABC A17P overlaps with the gene for the lung surfactant regulator ABC A3, and sequence homology analyses strongly suggest that ABC A3 is the progenitor gene of ABC A17P. In fact ABC A17 has 55.3% amino acid identity with ABC A3 (Ban et al., 2005; Piehler et al., 2006). In addition, an Abca3-like gene in the sea urchin genome, suAbca, is expressed in sperm and appears to be involved in shedding of cholesterol during sperm head maturation (Mengerink and Vacquier, 2002).

In the present study, we have obtained strong evidence indicating that ABC A17 is involved in sperm cholesterol efflux. The combination of NP40 and ABC A17 antibody produced a 40% inhibition of cholesterol efflux to lipid acceptor apoAI in sperm isolated from the corpus epididymidis. No effect was observed in sperm from the caput or cauda epididymidis. This inhibition was further increased when sperm were incubated in a cocktail containing both anti-ABC A17 and anti-ABC G1 antibodies. In fact, the combination of both antibodies produced a statistical significant inhibition of cholesterol efflux at the level of 25% in the corpus and 40% in the cauda epididymidis that was mainly attributed to the blockage of ABC G1. However, we cannot discard the possibility that both ABC
molecules operate synergistically.

Our findings indicate that ABCA17 mediated cholesterol efflux occurs exclusively in spermatozoa isolated from the corpus epididymis in presence of ApoA-I. Interestingly, ABCA1, ABCA7 and ABCG1 have also been shown to function in a regional specific manner in the epididymis (Morales et al., 2008). The physiological significance of the regional activity of the ABC transporters suggests that spermatozoa membrane remodelling takes place gradually during their maturation in the epididymis. The modification of spermatozoa cholesterol content during epididymal maturation has been investigated in several mammals, and a significant decrease of about 50% has been reported in ram, rat, hamster and mouse (reviewed by Saez et al., 2011). The cholesterol loss is usually accompanied by a decrease in the cholesterol/phospholipids ratio, an indicator of membrane fluidity, suggesting that spermatozoa increase their membrane fluidity as the sperm transit through the epididymis (reviewed by Saez et al., 2011). In the goat, however, the spermatozoa cholesterol content increases during epididymal maturation, whereas in boar, no significant changes were noticed (reviewed by Saez et al., 2011). Although little is known about the content of cholesterol in the plasma membrane of human spermatozoa, the fluidity of cauda epididymal sperm was shown to be higher than the one from caput epididymal sperm, suggesting a decrease in cholesterol content (reviewed by Saez et al., 2011).

In summary, the ABC transporter, ABCA17, is expressed on the head and flagellar midpiece of mouse spermatozoa. Treatment of spermatozoa with anti-ABCA17 antibody reduces cholesterol efflux to lipid acceptors suggesting that this transporter plays a role in the process of sterol efflux. The latter activity may render spermatozoa capable of interacting with an oocyte by inducing the acrosome reaction and hyperactivated motility that leads to fertilization.

References


ABC A17 expression in mouse testis


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